UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF CHEMICAL SAFETY AND POLLUTION PREVENTION

MEMORANDUM

DATE: **December 20, 2010**

SUBJECT: **2, 4-Dichlorophenoxyacetic Acid (2,4-D) - Report of the Endocrine Disruptor Review Team - Test Order #: EDSP–031001-120**

PC Code: 030001
Decision No.: N/A
Petition No.: N/A
Risk Assessment Type: N/A
TXR No.: 0055461
MRID No.: See Section V

DP Barcode: D377649 and D377658
Registration No.: N/A
Regulatory Action: N/A
Case No.: N/A
CAS No.: N/A
40 CFR: N/A

FROM: Greg Akerman, Ph.D.
Executive Secretary
Endocrine Disruptor Review Team

THROUGH: Karen Whitby, Ph.D., Co-Chair
Endocrine Disruptor Review Team
Office of Pesticide Programs
And
Gary Timm, Co-Chair
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Office of Science Coordination and Policy

TO: Katie Weyrauch
Chemical Review Manager
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SUMMARY CONCLUSIONS

Please find below a table that summarizes the Agency's conclusions regarding the submissions provided by the Test Order Recipient and the public in response to the Agency's Test Order for the screening assays included in the Endocrine Disruptor Screening Program (EDSP) Tier 1 battery.

This table summarizes the initial response of the Test Order Recipient(s) as well as the conclusion of the Office of Chemical Safety and Pollution Prevention (OCSPP) Endocrine Disruptor Review Team (EDRT).

Chemical: 2,4-D			PC Code: 031001		
		Test Order Recipient Response		Agency's Conclusions	
Guideline	**Assay**	**Will Generate New Data**	**Existing Data Cited**	**Does Cited Data Satisfy the Order**	**Rationale**
890.1100	Amphibian Metamorphosis Assay (Frog)	Yes	No	N/A	See Table 1 below.
890.1150	Androgen Receptor Binding (Rat Prostate)	No	Yes	No	See Table 2 below.
890.1200	Aromatase Assay (Human Recombinant)	No	Yes	No	See Table 3 below.
890.1250	Estrogen Receptor Binding	No	Yes	No	See Table 4 below.
890.1300	Estrogen Receptor Transcriptional Activation (Human Cell Line HeLa-9903)	No	Yes	No	See Table 5 below.
890.1350	Fish Short-Term Reproduction	Yes	No	N/A	See Table 6 below.
890.1400	Hershberger (Rat)	No	Yes	Yes	See Table 7 below.
890.1450	Female Pubertal (Rat)	No	Yes	Yes	See Table 8 below.
890.1500	Male Pubertal (Rat)	No	Yes	Yes	See Table 9 below.
890.1550	Steroidogenesis (Human Cell Line – H295R)	No	Yes	No	See Table 10 below.
890.1600	Uterotrophic (Rat)	No	Yes	Yes	See Table 11 below.

N/A = Not applicable; the Test Order Recipient has agreed to conduct this assay.

The Test Order Recipient will need to conduct the Tier 1 EDSP 890 Series Guideline assays identified in the table above that the submitter agreed to perform or those that are not satisfied by the OSRI submitted.

I. BACKGROUND

On October 29, 2009, the Agency began to issue test orders for the initial list of chemicals to be tested in the Endocrine Disruptor Screening Program (EDSP) Tier 1 battery under authority provided in section 408(p)(5) of the Federal Food, Drug, and Cosmetic Act (FFDCA). The EDSP Tier 1 screening data required to satisfy an order are due within 2 years of the date of issuance of the order. The policies and procedures the Agency will use for the initial screening of chemicals are described in FRN Vol. 74, No. 71 (April 15, 2009).

The Agency formed the Endocrine Disruptor Review Team (EDRT) to support OCSPP scientists in their review of "other scientifically relevant information"[1] that may be cited by Test Order Recipients or the public in response to EDSP Tier 1 test orders. The EDRT provides a centralized venue for the review of OSRI submitted in response to the EDSP Tier 1 test orders issued under 408(p) of FFDCA to screen pesticide chemicals for their potential to interact with the estrogen; androgen and thyroid (EAT) hormonal systems. The goal of the EDRT is to reach consistent, transparent and defensible conclusions on responses to the test orders for existing data cited and submitted to the Agency which are believed to be sufficient to satisfy part or all of the EDSP Tier 1 Test Order data requirements.

II. WEIGHT OF EVIDENCE EVALUATION OF THE OSRI

Section II of this document provides a summary of the Agency review of existing data cited as OSRI by either the Test Order Recipient or the public. Existing data may include data previously submitted to the Agency in support of a registration decision believed to be relevant to one or more of the assays in the test order. The cited study and its supporting data were considered relative to the Tier 1 EDSP assay for which they were cited. The Part 158 test guideline studies cited as OSRI are listed in bibliography section (Section V) of this report. The Agency conducted a weight-of-evidence determination of the significance of the data cited as OSRI by all sources (i.e., either the Test Order Recipient or the public). The synthesis of this analysis is presented in Section II which consists of eleven tables; there is a table for each of the eleven assays which comprise the Tier 1 EDSP battery. Studies evaluated by the Agency in drawing conclusions to accept or reject the OSRI rationale are presented in the table for each of the respective assays along with the Agency's rationale for the decision.

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[1] Current Working Definition: "Other scientifically relevant information" is information that informs the determination as to whether the substance may have an effect that is similar to an effect produced by a substance that interacts with the estrogen, androgen, and/or thyroid hormonal systems (e.g., information that identifies substances as having the potential to interact with the estrogen, androgen, and/or thyroid system(s); information demonstrating whether substances have an effect on the functioning of the endocrine system). OSRI may either be functionally equivalent to information obtained from the Tier 1 assays—that is, data from assays that perform the same function as EDSP Tier 1 assays—or may include data that provide information on a potential consequence or effect that could be due to effects on the estrogen, androgen or thyroid systems.

The EDRT's evaluations of the existing data cited in the OSRI are presented below in the following tables. Each of the tables provides the citations for existing data submitted to the Agency that were considered by the EDRT in their decision making. EDRT determined whether the cited/submitted data received from the Industry Task Force on 2,4-D Research Data (Test Order Recipient) and), People for the Ethical Treatment of Animals (PETA) and Physicians Committee for Responsible Medicine (PCRM) provided an accepted scientific method or protocol (and any other information relevant) to satisfy the requirements of the Test Order.

Section III of this memorandum contains a table that summarizes the endocrine-related findings in the studies cited in the submissions by the Test Order Recipient and the public that were considered in the EDRT's weight of evidence evaluation.

Section IV of this memorandum contains a table that lists studies cited in the submissions by the Test Order Recipient and the public that were not used in the EDRT's weight of evidence evaluation and provides the reasons for this decision.

Section V of this memorandum contains the bibliography of all cited data from all sources (Test Order Recipient(s) and public responses).

Table 1. Evaluation of Data Submitted in Relation to the Amphibian Metamorphosis Assay

Chemical: 2,4-D	**PC Code: 031001**

890.1100 - Amphibian Metamorphosis Assay (Frog)

1. EDSP Assay Endpoints[1]

Study Type / Literature Citation	MRID No.	Developmental Stage	Hind Limb Length	Snout-Vent Length	Wet Body Weight	Thyroid Histopathology
N/A	N/A	N/A	N/A	N/A	N/A	N/A

2. Summary of Study Findings:

Study Type / Literature Citation	MRID No.	Findings
N/A	N/A	N/A

3. Agency's Evaluation of the OSRI: N/A

4. Conclusion:

The Test Order Recipient has agreed to conduct this assay.

[1] -- = not measured; X = indicates the endpoint was measured in the assay but does not indicate whether or not it satisfies the data requirement of the test order. See section 3 below for a detailed explanation; N/A = not applicable

Table 2. Evaluation of Data Submitted in Relation to the Androgen Receptor Binding Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1150 - Androgen Receptor Binding Assay (Rat Prostate)

1. EDSP Assay Endpoints[1]

Study Type / Literature Citation	MRID No.	Binding Curve fit to Hills four-parameters are:			
		Top	Bottom	Slope	Log (IC_{50})
ToxCast program	N/A	--	--	--	--
Fang *et al.*, (2003)	48074110	--	--	--	x
Kim *et al.*, (2005)	N/A	--	--	--	--
Kojima *et al.*, (2004)	48033008	--	--	--	--
Orton *et al.*, (2009)	48041727	--	--	--	--
Extended 1-Generation Reproduction - Rat	47972101	--	--	--	--
2-Generation Reproduction - Rat	00150557	--	--	--	--
Developmental Toxicity - Acid - 2,4-D Rat	00130407	--	--	--	--
Developmental Toxicity – 2,4-D Salts & Esters – Rat [Charles *et al.*, (2001)]	45761204	--	--	--	--
Developmental Toxicity –2,4-D Acid Rabbit	41747601	--	--	--	--
Developmental Toxicity – 2,4-D Salts& Esters – Rabbit [Charles *et al.*, (2001)]	45761204	--	--	--	--
Chronic toxicity/Carcinogenicity – Rat	43612001	--	--	--	--
Carcinogenicity – Male Mouse	43879801	--	--	--	--
Carcinogenicity – Female Mouse	43597201	--	--	--	--
1981: Subchronic Oral toxicity - Rat	00102451	--	--	--	--
1991: Subchronic Oral Toxicity- 2,4-D Acid - Rat	41991501	--	--	--	--
Subchronic Oral Toxicity – 2,4-D Salts & Esters – Rat [Charles *et al.*, (1996)]	45761213	--	--	--	--
Subchronic Oral Toxicity -Mouse	41991502	--	--	--	--

Table 2. Evaluation of Data Submitted in Relation to the Androgen Receptor Binding Assay						
Chemical: 2,4-D			**PC Code: 031001**			
890.1150 - Androgen Receptor Binding Assay (Rat Prostate)						
Subchronic Inhalation Toxicity- Rat	47398701	--	--	--	--	
Avian Reproduction Study with Bobwhite Quail	45336401	--	--	--	--	
Epidemiology Studies (PETA OSRI)	N/A	--	--	--	--	

2. Summary of Study Findings:

Study Type / Literature Citation	MRID No.	Findings
ToxCast program	N/A	See discussion below in Section 3.
Epidemiology Studies (PETA OSRI)	N/A	See discussion below in Section 3.
Fang *et al.*, (2003)	48074110	See discussion below in Section 3.
Kim *et al.*, (2005)	N/A	See discussion below in Section 3.
Kojima *et al.*, (2004)	48033008	See discussion below in Section 3.
Orton *et al.*, (2009)	48041727	See discussion below in Section 3.
Part 158 studies cited above	See above	The cited *in vivo* Part 158 toxicity studies do not measure the potential of the chemical to bind to the androgen receptor

3. Agency's Evaluation of the OSRI:

The submission provided by the Test Order Recipient stated that "This assay provides information on the ability of the test compound to bind to the androgen receptor (AR). For 2,4-D, this has been tested in several different test systems. The ToxCast® assays developed under the auspices of the US EPA cited as OSRI and reviewed in Appendix V showed no evidence of 2,4-D interacting with the AR in a cell-based or cell-free system. AR binding/transactivation studies in the published literature fail to show positive responses to 2,4-D as summarized in Appendix III. Review of the reproductive and developmental toxicity, and subchronic and chronic toxicity and/or oncogenicity data from regulatory studies for 2,4-D cited as OSRI do not show any consistent patterns of effects suggesting binding of 2,4-D to the AR at doses below the KMD. There is no evidence that findings observed at high doses well above the KMD were related to direct interaction with the androgen system. Based on the extensive data available and cited as OSRI, including *in vitro* assays specifically addressing the potential AR binding and/or transactivation potential of 2,4-D, the 2,4-D Task Force requests a waiver from an additional *in vitro* assay of AR binding."

Table 2. Evaluation of Data Submitted in Relation to the Androgen Receptor Binding Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1150 - Androgen Receptor Binding Assay (Rat Prostate)

The PETA comments submitted to the Agency in response to the EDSP screening order issued for 2,4-D stated that "In summary, 2,4-D is an extremely well-studied chemical. Its endocrine disrupting potential has already been addressed in mammalian subchronic, reproductive and developmental studies as well as in numerous *in vitro* assays, in chronic studies in birds, fish and reptiles and in epidemiologic studies. Further, a study designed in consultation with the EPA to definitively address remaining endocrine-sensitive endpoints is nearing completion. There is no need for further testing under the EDSP."

On review of the OSRI submitted, the Agency noted a number of deficiencies and therefore has outstanding questions about the potential for 2,4-D to interact with the androgen receptor.

- Although the cited ToxCast data may be appropriate for use in priority setting, ToxCast *in vitro* assays cannot at this time be considered acceptable alternatives to the EDSP Tier 1 *in vitro* assays. Thus this information is not considered sufficient to satisfy the Test Order requirement for the Androgen Receptor Binding Assay using Guideline 890.1150 (Kavlock and Zenick, 2010).

- Fang *et al.*, (2003) reported data for each competitor and R1881 (standard AR ligand) standard curve were plotted as [^{3}H]R1881 bound (relative to the standard) vs. molar concentrations. 2,4-D was adequately tested at concentrations from 4.28 x 10^{-9} to 4.28 x 10^{-4} M. All assays were run in duplicate with at least two replications. Appropriate reference chemicals were used in the study. The IC_{50} (50% inhibition of R1881 standard) values were reported and the relative binding affinities (RBA) were calculated for each chemical. Based on the RBA values, the chemicals were classified as strong binders, moderate binders, weak binders or inactive (non-binders). 2,4-D was classified in this study as a non-binder. The authors compared the recombinant AR binding data generated in this study to a published data set that was generated using the rat ventral prostate cytosol AR method similar to the one used in the EDSP Tier 1 assay. There were 20 chemicals in common between the two data sets. The results from the two methods were generally comparable with the recombinant AR method demonstrating adequate sensitivity to identify strong, moderate and weak androgen receptor binders. Radiolabeled [^{3}H]-R1881 is used to measure competitive AR binding (similar to the Tier 1 assay). No concentration response information were available for 2.4-D to substantiate the conclusion that the study was negative. If these data were provided, EPA would reconsider the acceptability of this study to meet the requirements of the test order.

Table 2. Evaluation of Data Submitted in Relation to the Androgen Receptor Binding Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1150 - Androgen Receptor Binding Assay (Rat Prostate)

- Kim *et al.*, (2005) conducted an AR receptor binding assay; however, a whole cell assay was used rather (COS-1 cells transiently transfected with human AR) than the cell-free receptor required in 890.1150. Due to the lower sensitivity of the whole cell binding assay as, Kim does not meet Guideline 890.1150.

- Kojima *et al.*, (2004) conducted a transcriptional activation assay and tested a narrower range of concentrations than the OPPTS 890.1150 Guideline requires. Transcriptional activation assays do not measure receptor binding and involve post-binding events; therefore, they are not an adequate substitute for receptor binding assays.

- Orton *et al.*, (2009) conducted the yeast androgen screen with 2,4-D. 2,4-D was reported to be negative. No concentration response data were reported to substantiate the negative result. In addition, is particularly important to substantiate that the chemical is capable of crossing the cell wall for a negative result in a yeast assay, as some chemicals do not cross the cell wall.

- The PETA submission provided a brief review of human studies that addressed potential reproductive and developmental toxicity of 2, 4-D based on the exposure of Vietnam veterans to Agent Orange. The conclusion was that the veterans who had greater potential exposure to Agent Orange did not have an increased risk of fathering babies with major structural birth defects. The submission also noted that, even for pesticide applicators and the general population in an agricultural region of Minnesota, a more detailed cross-sectional analysis of this area showed no statistically significant correlation between 2, 4-D use and excess adverse birth effects. The Agency believes that overall, prospective cohort studies still need to be conducted to confirm or test the hypothesis generated by these studies. Further, additional detail is needed on exposures of the subjects to drugs and chemicals which may have an adverse effect on the male reproductive system that can contribute confounding effects in the outcome and interpretation of these data. While these data provide additional information that upon further investigation may contribute to hazard characterization, they do not provide confirmed or confident linkages between human exposure to 2,4-D and the potential for interaction with the androgen receptor.

4. Conclusion:

Based on the deficiencies and outstanding questions discussed above, the data cited as OSRI did not satisfy the requirement for the Androgen Receptor Binding Assay using Guideline 890.1150. EPA will reconsider this conclusion if supporting data for Fang et al is provided.

[1] -- = not measured; X = indicates the endpoint was measured in the assay but does not indicate whether or not it satisfies the data requirement of the test order. See section 3 below for a detailed explanation; N/A = not applicable.

Table 3. Evaluation of Data Submitted in Relation to the Aromatase (Human Recombinant) Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1200 - Aromatase Assay (Human Recombinant)

1. EDSP Assay Endpoints[1]

Study Type / Literature Citation	MRID No.	^3H_2O measured	Estrone measured
ToxCast program	N/A	--	--
Crain *et al.*, (1997)	N/A	--	--
Crain *et al.*, (1999)	N/A	--	--
Spiteri *et al.*, (1999)	N/A	--	--
Extended 1-Generation Reproduction - Rat	47972101	--	--
2-Generation Reproduction - Rat	00150557	--	--
Developmental Toxicity - Acid - 2,4-D Rat	00130407	--	--
Developmental Toxicity – 2,4-D Salts & Esters – Rat [Charles *et al.*, (2001)]	45761204	--	--
Developmental Toxicity –2,4-D Acid Rabbit	41747601	--	--
Developmental Toxicity – 2,4-D Salts& Esters – Rabbit [Charles *et al.*, (2001)]	45761204	--	--
Chronic toxicity/Carcinogenicity – Rat	43612001	--	--
Carcinogenicity – Male Mouse	43879801	--	--
Carcinogenicity – Female Mouse	43597201	--	--
1981: Subchronic Oral toxicity - Rat	00102451	--	--
1991: Subchronic Oral Toxicity- 2,4-D Acid - Rat	41991501	--	--
Subchronic Oral Toxicity – 2,4-D Salts & Esters - Rat	45761213	--	--
Subchronic Oral Toxicity -Mouse	41991502	--	--
Subchronic Inhalation Toxicity- Rat	47398701	--	--
Avian Reproduction Study with Bobwhite Quail	45336401	--	--
Epidemiology Studies (PETA OSRI)	N/A	--	--

Table 3. Evaluation of Data Submitted in Relation to the Aromatase (Human Recombinant) Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1200 - Aromatase Assay (Human Recombinant)

2. Summary of Study Findings:

Study Type / Literature Citation	MRID No.	Findings
ToxCast program	N/A	See discussion below in Section 3.
Crain *et al.*, (1997)	N/A	See discussion below in Section 3.
Crain *et al.*, (1999)	N/A	See discussion below in Section 3.
Spiteri e*t al.*, (1999)	N/A	See discussion below in Section 3.
Epidemiology Studies (PETA OSRI)	N/A	See discussion below in Section 3.
Part 158 studies cited above	See above	The cited *in vivo* Part 158 studies do not measure the potential of the chemical to alter aromatase enzyme activity.

3. Agency's Evaluation of the OSRI:

The submission provided by the Test Order Recipient stated that "This assay focuses on whether the test compound inhibits aromatase activity in an *in vitro* system. The ToxCast® assay developed under the auspices of the US EPA cited as OSRI and reviewed in Appendix IV showed no evidence of aromatase inhibition. Studies of aromatase inhibition in the gonad–adrenal–mesenephros complex or liver in American alligators (Crain *et al.*, 1997 and Crain *et al.* 1999) showed no adverse effects from 2,4-D exposures. A follow-up study by Spiteri *et al.* (1999) found embryonic exposure to 2,4-D did not cause significant histopathological alterations in gonadal structure of hatchling American alligators. Further, a review of the in vivo mammalian regulatory toxicity data, discussed in detail below, shows no evidence of anti-estrogenicity (which would be predicted if the aromatase enzyme were inhibited). Based on the extensive data available and cited as OSRI, including a specific *in vitro* assay of aromatase inhibition, the 2,4-D Task Force requests a waiver from an additional Aromatase assay."

The PETA comments submitted to the Agency in response to the EDSP screening order issued for 2,4-D stated that "In summary, 2,4-D is an extremely well-studied chemical. Its endocrine disrupting potential has already been addressed in mammalian subchronic, reproductive and developmental studies as well as in numerous *in vitro* assays, in chronic studies in birds, fish and reptiles and in epidemiologic studies. Further, a study designed in consultation with the EPA to definitively address remaining endocrine-sensitive endpoints is nearing completion. There is no need for further testing under the EDSP."

Table 3. Evaluation of Data Submitted in Relation to the Aromatase (Human Recombinant) Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1200 - Aromatase Assay (Human Recombinant)

On review of the OSRI submitted, the Agency noted a number of deficiencies and therefore has outstanding questions about the potential for 2, 4-D to alter the aromatase enzyme.

- Although the cited ToxCast data may be appropriate for use in priority setting, ToxCast *in vitro* assays cannot at this time be considered acceptable alternatives to the EDSP Tier 1 *in vitro* assays. Thus this information is not considered sufficient to satisfy the Test Order requirement for the Aromatase Assay using Guideline 890.1200 (Kavlock and Zenick, 2010).

- Crain *et al.,* (1997) studied the aromatase activity in the gonadal-adrenal-mesenephros complex taken from female alligators which had been exposed *in ovo* to various chemicals to investigate the mechanism by which a pesticide contaminated lake affected sex-determination. Exposure to 2,4-D did not affect aromatase. This assay measures the effect of a chemical on the induction of aromatase in hatchlings due to *in ovo* exposure. While the authors demonstrated that a strong inhibitor of aromatase could reduce aromatase activity, the Agency has concerns about the variability and sensitivity of the assay procedure and whether cytotoxicity has been adequately accounted for. This assay resembles to some extent the sliced testes assay which EPA attempted to validate, which failed to validate on the basis of high variability and lack of ability to determine cytotoxicity to the cells responsible for steroid synthesis in the tissue slices. This study may provide ancillary information but cannot replace 890.1200.

- Crain *et al.,* (1999) studied hepatic aromatase activity using liver slices from hatchling alligators with androstenedione as the substrate and tritiated water as the measured reaction product. This experiment determined the effect of pesticides on aromatase levels in tissue when hatchlings were exposed *in ovo*. This study uses the same methodology as Crain 1997 and has the same limitations. Thus, it is not acceptable as a substitute for 890.1200.

- Spiteri *et al.,* (1999) conducted the same type of study as Crain *et al.* (1997 and 1999), and has the same limitations.

- The PETA submission provided a brief review of a few human studies that addressed potential reproductive and developmental toxicity of 2,4-D based on the exposure of Vietnam veterans to Agent Orange. The conclusion was that the veterans who had greater potential exposure to Agent Orange did not have an increased risk of fathering babies with major structural birth defects. The submission also stated that, even for pesticide applicators and the general population in an agricultural region of Minnesota, a more detailed cross-sectional analysis of this area showed no statistically significant correlation between 2,4-D use and excess adverse birth effects. EPA believes that overall, prospective cohort studies still need to be conducted to confirm or test the

Table 3. Evaluation of Data Submitted in Relation to the Aromatase (Human Recombinant) Assay	
Chemical: 2,4-D	**PC Code: 031001**
890.1200 - Aromatase Assay (Human Recombinant)	
hypothesis generated by these studies. Additional detail is needed on exposures of the subjects to drugs and chemicals which may have an adverse effect on the male reproductive system that can contribute confounding effects in the outcome and interpretation of these data. While these data provide additional information that upon further investigation may contribute to hazard characterization, they do not provide confirmed or confident linkages between human exposure to 2,4-D and the potential for interference with the aromatase enzyme.	
4. Conclusion: Based on the deficiencies and outstanding questions discussed above, the data cited as OSRI did not satisfy the requirement for the Aromatase Assay using Guideline 890.1200.	

[1] -- = not measured; X = indicates the endpoint was measured in the assay but does not indicate whether or not it satisfies the data requirement of the test order. See section 3 below for a detailed explanation; N/A = not applicable

Table 4. Evaluation of Data Submitted in Relation to the Estrogen Receptor Binding Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1250 - Estrogen Receptor Binding Assay

1. EDSP Assay Endpoints[1]

Study Type / Literature Citation	MRID No.	Binding Curve fit to Hills four-parameters are:			
		Top	Bottom	Slope	Log(IC_{50})
ToxCast Program	N/A	--	--	--	--
Blair *et al.* (2000)	48161802	--	--	--	--
Hurst and Sheahan (2003)	48242003	--	--	--	--
Hwang (2002)	N/A	--	--	--	--
Jung *et al.*, (2004)	N/A	--	--	--	--
Jungbauer and Beck (2002)	N/A	--	--	--	--
Lee *et al.* (2006)	N/A	--	--	--	--
Lin and Garry (2000)	48033008	--	--	--	--
Kojima *et al.*, (2004)	48074114	--	--	--	--
Lemaire *et al.*, (2006)	48041723	--	--	--	--
Nishihara *et al.*, (2000)	48041727	--	--	--	--
Orton *et al.*, (2009)	N/A	--	--	--	--
Petit *et al.*, (1997)	48074102	--	--	--	--
Soto *et al.*, (1995)	N/A	--	--	--	--
Vonier *et al.*, (1996)	48161802	--	--	--	--
Extended 1-Generation Reproduction - Rat	47972101	--	--	--	--
2-Generation Reproduction - Rat	00150557	--	--	--	--
Developmental Toxicity - Acid - 2,4-D Rat	00130407	--	--	--	--
Developmental Toxicity – 2,4-D Salts & Esters – Rat [Charles *et al.*, (2001)]	45761204	--	--	--	--

Table 4. Evaluation of Data Submitted in Relation to the Estrogen Receptor Binding Assay						
Chemical: 2,4-D			**PC Code: 031001**			
890.1250 - Estrogen Receptor Binding Assay						
Developmental Toxicity –2,4-D Acid Rabbit	41747601	--	--	--	--	
Developmental Toxicity – 2,4-D Salts& Esters – Rabbit [Charles *et al.*, (2001)]	45761204	--	--	--	--	
Chronic toxicity/Carcinogenicity – Rat	43612001	--	--	--	--	
Carcinogenicity – Male Mouse	43879801	--	--	--	--	
Carcinogenicity – Female Mouse	43597201	--	--	--	--	
1981: Subchronic Oral toxicity - Rat	00102451	--	--	--	--	
1991: Subchronic Oral Toxicity- Rat	41991501	--	--	--	--	
Subchronic Oral Toxicity– 2,4-D Salts & Esters - Rat	45761213	--	--	--	--	
Subchronic Oral Toxicity -Mouse	41991502	--	--	--	--	
Subchronic Inhalation Toxicity- Rat	47398701	--	--	--	--	
Avian Reproduction Study with Bobwhite Quail	45336401	--	--	--	--	
Epidemiology Studies (PETA OSRI)	N/A	--	--	--	--	
2. Summary of Study Findings:						
Study Type / Literature Citation	**MRID No.**	**Findings**				
ToxCast Program	N/A	See discussion below in Section 3.				
Blair *et al.* (2000)	48161802	See discussion below in Section 3.				
Hurst and Sheahan (2003)	48242003	See discussion below in Section 3.				
Hwang (2002)	N/A	See Section IV of this report.				
Jung *et al.*, (2004)	N/A	See discussion below in Section 3.				
Jungbauer and Beck (2002)	N/A	See discussion below in Section 3.				
Lee *et al.* (2006)	N/A	See discussion below in Section 3.				
Lin and Garry (2000)	48033008	See discussion below in Section 3.				
Kojima *et al.*, (2004)	48074114	See discussion below in Section 3.				
Lemaire *et al.*, (2006)	48041723	See discussion below in Section 3.				

Table 4. Evaluation of Data Submitted in Relation to the Estrogen Receptor Binding Assay		
Chemical: 2,4-D		**PC Code: 031001**
890.1250 - Estrogen Receptor Binding Assay		
Nihihara *et al.*, (2000)	48041727	See discussion below in Section 3.
Orton *et al.*, (2009)	N/A	See discussion below in Section 3.
Petit *et al.*, (1997)	48074102	See discussion below in Section 3.
Soto *et al.*, (1995)	N/A	See discussion below in Section 3.
Vonier *et al.*, (1996)	48161802	See discussion below in Section 3.
Epidemiology Studies (PETA OSRI)	N/A	See discussion below in Section 3.
Part 158 studies cited above	See above	The cited *in vivo* Part 158 studies do not measure the potential of the chemical to bind to the estrogen receptor.

3. Agency's Evaluation of the OSRI:

The submission provided by the Test Order Recipient stated that "This assay provides information on the ability of the test compound to bind to the estrogen receptor (ER). For 2,4-D, this has been tested in several different test systems. The ToxCast® assays developed under the auspices of the US EPA cited as OSRI and reviewed in Appendix IV showed no evidence for 2,4-D of binding to ERα or ERβ in a cell-free system. ER binding studies have also been identified in the published literature as reviewed in Appendix III. These assays also fail to show 2,4-D binding to the ER, with a single exception. Further, review of the reproductive and developmental toxicity, subchronic and chronic toxicity and/or oncogenicity data from regulatory studies for 2,4-D cited as OSRI do not show any consistent patterns of effects suggesting that 2,4-D binds to the rodent ER. Based on the extensive data available and cited as OSRI, including *in vitro* assays specifically addressing the potential ER binding of 2,4-D, the 2,4-D Task Force requests a waiver from an additional *in vitro* assay of ER binding potential."

"The PETA comments submitted to the Agency in response to the EDSP screening order issued for 2,4-D stated that "In summary, 2,4-D is an extremely well-studied chemical. Its endocrine disrupting potential has already been addressed in mammalian subchronic, reproductive and developmental studies as well as in numerous *in vitro* assays, in chronic studies in birds, fish and reptiles and in epidemiologic studies. Further, a study designed in consultation with the EPA to definitively address remaining endocrine-sensitive endpoints is nearing completion. There is no need for further testing under the EDSP."

Table 4. Evaluation of Data Submitted in Relation to the Estrogen Receptor Binding Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1250 - Estrogen Receptor Binding Assay

On review of the OSRI submitted, the Agency noted a number of deficiencies and therefore has outstanding questions about the potential for 2,4-D to interact with the estrogen receptor:

- Although the cited ToxCast data may be appropriate for use in priority setting, ToxCast *in vitro* assays cannot at this time be considered acceptable alternatives to the EDSP Tier 1 *in vitro* assays. Thus this information is not considered sufficient to satisfy the Test Order requirement for the Estrogen Receptor Binding Assay using Guideline 890.1250 (Kavlock and Zenick, 2010).

- Blair *et al.* (2000) tested 2,4-D in an ER assay using rat uterine cytosol as the receptor source. Test chemicals were initially tested at two high concentrations. Positive chemicals were retested to obtain a concentration-response curve. 2.4-D was negative, but no data were supplied to substantiate this conclusion. In addition, only two concentrations were tested, which is generally insufficient to preclude false negatives/positives. Test Guideline 890.1250 recommends that a concentration response curve be generated. Therefore, this study does not meet test order requirements.

- Hurst and Sheahan (2003) conducted a Yeast Estrogen Screen with 2,4-D. The results were reported to be negative. As noted elsewhere, a yeast based screen may be subject to false negatives due to the inability of the test substance to permeate the cell wall. In addition, the study does not measure binding and no data were supplied to substantiate this conclusion. Therefore this study does not fulfill the requirements of the test order.

- Hwang (2002) - See Section IV of this report.

- Jung *et al.*, (2004) examined the anti-estrogenic activity of 52 chemicals using the following approach. Chemicals were first tested for anti-estrogenicicty in the yeast two hybrid system transfected with a rat ER receptor. Chemicals that were identified as anti-estrogens in this assay were then tested in a transcriptional activation assay using MCF-7 cells, and an ER binding assay using fluorescence polarization. This study has several disadvantages. First the gatekeeper was the yeast assay for anti-estrogenic activity, so no additional testing would be conducted if a chemical were not identified as an anti-estrogen by the yeast assay. Because 2,4-D was not positive in the yeast assay it appears that it was never tested in the binding assay, but the article is not absolutely clear on this point. No ER binding data were reported for 2,4-D.

Table 4. Evaluation of Data Submitted in Relation to the Estrogen Receptor Binding Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1250 - Estrogen Receptor Binding Assay

- Jungbauer and Beck (2002) tested 2,4-D in a yeast based system. The concentrations were not identified nor were data provided. This study was not cited as OSRI by the submitter due to the limitations of yeast-based assays. See Section IV of this report.

- Lee *et al.* (2006) used a yeast based two-hybrid system. 2,4-D was reported to be positive in this system with binding over the range 2.09×10^{-4} to 5.42×10^{-6} M. This study was not cited as OSRI by the submitter due to the limitations of yeast-based assays.

- Lin and Garry (2000) tested 2,4-D in an MCF-7 cell proliferation assay. Technical grade 2,4-D was positive in the assay, but the reagent grade was negative. A cell proliferation assay does not measure receptor binding and may be affected by factors other than binding with the receptor. It may therefore be useful ancillary information, but cannot satisfy the requirements of the test order.

- Kojima *et al.*, (2004) tested 2,4-D (> 95% purity) in CHO-K1 cell transcriptional activation assay and reported negative results. No data were provided to substantiate this result. Furthermore, transcriptional activation assays do not measure receptor binding and involve post-binding events; therefore, they are not an adequate substitute for receptor binding assays.

- Lemaire *et al.*, (2006) tested 49 pesticides in HeLa cells transfected with ERα and ERβ. Substances were tested first at 10 μM. Positive substances were re-tested to determine a dose response curve and calculate an EC50. 2,4-D was reported negative with a percent activity in ERα and ERβ of 8.4 ± 1.4 and 10.2 ± 2.1 which was not statistically different from the solvent DMSO (9.3 ± 1.3 and 10.8 ± 1.6). There are two concerns with this study: first, testing was only conducted at a single concentration, which is insufficient because testing at a single concentration increases the chances of false negatives and false positives. Second, the study did not measure binding to the receptor.

- Nishihara *et al.*, (2000) conducted a yeast two hybrid assay. The limitations of yeast based assays were recognized by the submitter and the study was not considered to be OSRI.

- Orton *et al.*, (2009) conducted a yeast estrogen screen on 2,4-D. It was reported negative; however, no data were provided, and the assay is subject to the limitations of yeast-based assays described above.

Table 4. Evaluation of Data Submitted in Relation to the Estrogen Receptor Binding Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1250 - Estrogen Receptor Binding Assay

- Petit *et al.*, (1997) tested 2,4-D in three different in vitro assays. It was tested in a yeast transactivation assay over a range of 10^{-8} to 10^{-4} M in which the yeast was transfected with rainbow trout ERα using a β-galactosidase reporter. Similarly a competitive binding assay was run using yeast cells and rainbow trout ERα. It was also tested for its ability to induce vitellogenin mRNA in a primary rainbow trout hepatocyte culture. The assay results were reported as follows: β-Gal expression in the TA assay = 17.95%, 8.0% Vg induction, and fold induction needed to displace 50% of bound E2 > 10,000. All results were described as negative. 2,4-D was tested at four concentrations in the whole cell extract receptor binding assay. It appeared that it displaced 20% of the E2 which would qualify it as negative, but the lack of experimental details and the need to interpret the results from a rather difficult to read diagram makes this study unacceptable to satisfy the requirements of the test order. EPA would reconsider this conclusion if more information were supplied. The transactivation and vitellogenin assays do not measure binding and are therefore not a substitute for the binding assay.

- Soto *et al.*, (1995) conducted an MCF-7 cell prolioferation assay. The results were described as negative, but no data were provided to substantiate this result. A cell proliferation assay could supplement the information provided by a binding study, but not substitute for it because it does not measure binding directly and other factors besides binding may stimulate proliferation.

- Vonier *et al.*, (1996) tested 2,4-D in a competitive binding assay using ER derived from alligator oviducts. 2,4-D was reported to be a non-binder, but no data were shown to substantiate this conclusion and the purity and range over which 2,4-D was tested were not specified. If the data were submitted, EPA would reconsider its conclusion.

- The PETA submission provided a brief review of a few human studies that addressed potential reproductive and developmental toxicity of 2,4-D based on the exposure of Vietnam veterans to Agent Orange. The conclusion was that the veterans who had greater potential exposure to Agent Orange did not have an increased risk of fathering babies with major structural birth defects. In fact, even for pesticide applicators and the general population in an agricultural region of Minnesota, a more detailed cross-sectional analysis of this area showed no statistically significant correlation between 2,4-D use and excess adverse birth effects. The Agency believes that overall, prospective cohort studies still need to be conducted to confirm or test the hypothesis generated by these studies. In addition, additional detail is needed on exposures of the subjects to drugs and chemicals which may have an adverse effect on the male reproductive system that can contribute confounding effects in the outcome and interpretation of these data. While these data provide additional information that upon further investigation may contribute to hazard characterization, they do not provide confirmed or confident linkages between human exposure to 2,4-D and the potential for interaction with the

Table 4. Evaluation of Data Submitted in Relation to the Estrogen Receptor Binding Assay	
Chemical: 2,4-D	**PC Code: 031001**
890.1250 - Estrogen Receptor Binding Assay	

estrogen receptor.

- None of the cited Part 158 studies measure binding of the chemical to the estrogen receptor (ER), which is the information that would be obtained by the Tier 1 ER binding assay. The argument presented in the explanations submitted to the Agency was that no estrogenic effects were seen in any of the cited studies. There was no substantive explanation explaining why the lack of any effect in these studies should be considered evidence that binding to the estrogen receptors does not occur. A lack of effect on potentially receptor-mediated endpoints in the mammalian *in vivo* studies cited does not necessarily demonstrate the absence of an interaction with the receptors in other species. Because chemicals that bind to a receptor but do not cause an effect in mammals may nevertheless show effects in other species, it is important to have information concerning direct interaction with the estrogen receptor.

4. Conclusion: Based on the deficiencies and outstanding questions listed above, the data cited as OSRI did not satisfy the requirement for the Estrogen Receptor Binding Assay using Guideline 890.1250.

[1] -- = not measured; X = indicates the endpoint was measured in the assay but does not indicate whether or not it satisfies the data requirement of the test order. See section 3 below for a detailed explanation; N/A = not applicable

Table 5. Evaluation of Data Submitted in Relation to the Estrogen Receptor Transcriptional Activation Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1300 - Estrogen Receptor Transcriptional Activation Assay (Human Cell Line HeLa-9903)

1. EDSP Assay Endpoints[1]

Study Type / Literature Citation	MRID No.	Bioluminescence measurements:		
		EC50	PC50	PC10
ToxCast Program	N/A	--	--	--
Blair *et al.* (2000)	48161802	--	--	--
Hurst and Sheahan (2003)	48242003	--	--	--
Hwang (2002)	N/A	--	--	--
Jung *et al.*, (2004)	N/A	--	--	--
Jungbauer and Beck (2002)	N/A	--	--	--
Lee *et al.* (2006)	N/A	--	--	--
Lin and Garry (2000)	N/A	--	--	--
Kojima *et al.*, (2004)	48033008	--	--	--
Lemaire *et al.*, (2006)	48074114	--	--	--
Nishihara *et al.*, (2000)	48041723	--	--	--
Orton *et al.*, (2009)	48041727	--	--	--
Petit *et al.*, (1997)	N/A	--	--	--
Soto *et al.*, (1995)	48074102	--	--	--
Vonier *et al.*, (1996)	N/A	--	--	--
Extended 1-Generation Reproduction - Rat	47972101	--	--	--
2-Generation Reproduction - Rat	00150557	--	--	--
Developmental Toxicity - Acid - 2,4-D Rat	00130407	--	--	--
Developmental Toxicity – 2,4-D Salts & Esters – Rat [Charles *et al.*, (2001)]	45761204	--	--	--
Developmental Toxicity –2,4-D Acid Rabbit	41747601	--	--	--
Developmental Toxicity – 2,4-D Salts& Esters – Rabbit [Charles *et al.*, (2001)]	45761204	--	--	--

Table 5. Evaluation of Data Submitted in Relation to the Estrogen Receptor Transcriptional Activation Assay				
Chemical: 2,4-D		**PC Code: 031001**		
890.1300 - Estrogen Receptor Transcriptional Activation Assay (Human Cell Line HeLa-9903)				
Chronic toxicity/Carcinogenicity – Rat	43612001	--	--	--
Carcinogenicity – Male Mouse	43879801	--	--	--
Carcinogenicity – Female Mouse	43597201	--	--	--
1981: Subchronic Oral toxicity - Rat	00102451	--	--	--
1991: Subchronic Oral Toxicity- Rat	41991501	--	--	--
Subchronic Oral Toxicity– 2,4-D Salts & Esters - Rat	45761213	--	--	--
Subchronic Oral Toxicity -Mouse	41991502	--	--	--
Subchronic Inhalation Toxicity- Rat	47398701	--	--	--
Avian Reproduction Study with Bobwhite Quail	45336401	--	--	--
Epidemiology Studies (PETA OSRI)	N/A	--	--	--
2. Summary of Study Findings:				
Study Type / Literature Citation	**MRID No.**	**Findings**		
ToxCast Program	N/A	See discussion below in Section 3.		
Blair *et al.* (2000)	48161802	See discussion below in Section 3.		
Hurst and Sheahan (2003)	48242003	See discussion below in Section 3.		
Hwang (2002)	N/A	See Section IV of this report.		
Jung *et al.*, (2004)	N/A	See discussion below in Section 3.		
Jungbauer and Beck (2002)	N/A	See discussion below in Section 3.		
Lee *et al.* (2006)	N/A	See discussion below in Section 3.		
Lin and Garry (2000)	48033008	See discussion below in Section 3.		
Kojima *et al.*, (2004)	48074114	See discussion below in Section 3.		
Lemaire *et al.*, (2006)	48041723	See discussion below in Section 3.		
Nishihara *et al.*, (2000)	48041727	See discussion below in Section 3.		
Orton *et al.*, (2009)	N/A	See discussion below in Section 3.		
Petit *et al.*, (1997)	48074102	See discussion below in Section 3.		
Soto *et al.*, (1995)	N/A	See discussion below in Section 3.		

Table 5. Evaluation of Data Submitted in Relation to the Estrogen Receptor Transcriptional Activation Assay		
Chemical: 2,4-D		**PC Code: 031001**
890.1300 - Estrogen Receptor Transcriptional Activation Assay (Human Cell Line HeLa-9903)		
Vonier *et al.*, (1996)	48161802	See discussion below in Section 3.
Epidemiology Studies (PETA OSRI)	N/A	See discussion below in Section 3.
Part 158 studies cited above	See above	These *in vivo* Part 158 studies do not measure the potential of the chemical to transactivate the ER.

3. Agency's Evaluation of the OSRI:

The submission provided by the Test Order Recipient stated that "This assay evaluates the potential of the test compound to transactivate the ER. The ToxCast® assays developed under the auspices of the US EPA cited as OSRI and reviewed in Appendix IV showed no evidence for 2,4-D interacting with ERα or ERβ in a cell based system. ER transactivation studies in the published literature fail to show positive response to 2,4-D, as summarized in Appendix III. The in vitro data across assays shows the overall response for binding to, activating or hindering ER activity is negative. As noted above, review of the reproductive and developmental toxicity, and subchronic and chronic toxicity and/or oncogenicity data from regulatory studies for 2,4-D cited as OSRI do not show any consistent patterns of effects suggesting interaction with the estrogen receptor (and no effects suggesting such an interaction below the KMD). One juvenile fish study suggests 2,4-D may increase vitellogenin (Xie *et al.*, 2005); this study suffered from significant endpoint response variability and the findings were inconsistent with *in vitro* studies suggesting a lack of ER binding or activation in trout (Petit *et al.*, 1997). The potential for this interaction will be further explored in the fish short-term reproduction assay which the 2,4-D Task Force plans to conduct. Based on the extensive data available and cited as OSRI, including in vitro assays specifically addressing the potential ER transactivation by 2,4-D, the 2,4-D Task Force requests a waiver from an additional *in vitro* assay of ER transcriptional activation."

The PETA comments submitted to the Agency in response to the EDSP screening order issued for 2,4-D stated that "In summary, 2,4-D is an extremely well-studied chemical. Its endocrine disrupting potential has already been addressed in mammalian subchronic, reproductive and developmental studies as well as in numerous *in vitro* assays, in chronic studies in birds, fish and reptiles and in epidemiologic studies. Further, a study designed in consultation with the EPA to definitively address remaining endocrine-sensitive endpoints is nearing completion. There is no need for further testing under the EDSP."

On review of the OSRI submitted, the Agency noted a number of deficiencies and therefore has outstanding questions about the potential for 2,4-D to transactivate the estrogen receptor.

Table 5. Evaluation of Data Submitted in Relation to the Estrogen Receptor Transcriptional Activation Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1300 - Estrogen Receptor Transcriptional Activation Assay (Human Cell Line HeLa-9903)

- Although the cited ToxCast data may be appropriate for use in priority setting, ToxCast *in vitro* assays cannot at this time be considered acceptable alternatives to the EDSP Tier 1 *in vitro* assays. Thus this information is not considered sufficient to satisfy the Test Order requirement for the Estrogen Receptor Transactivation Assay using Guideline 890.1300 (Kavlock and Zenick, 2010).

- Blair *et al.* (2000) tested 2,4-D in an ER assay using rat uterine cytosol as the receptor source. Test chemicals were initially tested at two high concentrations. Positive chemicals were retested to obtain a concentration-response curve. There are several problems with accepting this study in lieu of 890.1300: It is a receptor binding assay, not a transcriptional activation assay. Receptor binding assays cannot substitute for a transcriptional activation assay because it does not demonstrate consequences of binding and cannot differentiate between agonists and antagonists. Second, it would not suffice for a receptor binding assay since only two concentrations were tested therefore a concentration response curve cannot be generated. Third, no data were supplied to substantiate the conclusion that 2,4-D was negative in this assay.

- Hurst and Sheahan (2003) conducted a Yeast Estrogen Screen with 2,4-D. The results were reported to be negative. As noted elsewhere, a yeast-based screen may be subject to false negatives due to the inability of the test substance to permeate the cell wall. No data were submitted to demonstrate that 2,4-D could penetrate the cell wall or to otherwise substantiate this negative results. Therefore this study does not fulfill the requirements of the test order.

- Hwang (2002) - See Section IV of this report.

- Jung *et al.*, (2004) examined the anti-estrogenic activity of 52 chemicals using the following approach. Chemicals were first tested for anti-estrogenicity in the yeast two hybrid system transfected with a rat ER receptor. Chemicals that were identified as anti-estrogens in this assay were then tested in a transcriptional activation assay using MCF-7 cells, and an ER binding assay using fluorescence polarization. Although this study could be considered to be complementary to 890.1200, it fails to meet the objective of 890.1200 because it was only designed to detect anti-estrogens whereas 890.1300 is designed to detect estrogens. Because only chemicals that tested positive in the yeast anti-estrogen assay were subjected to further testing, 2-4-D was only evaluated in the yeast assay. As noted above, yeast assays may give rise to false negatives because some chemicals may fail to cross the cell wall, and no data were provided to demonstrate that 2,4-D was able to penetrate the cell wall.

Table 5. Evaluation of Data Submitted in Relation to the Estrogen Receptor Transcriptional Activation Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1300 - Estrogen Receptor Transcriptional Activation Assay (Human Cell Line HeLa-9903)

- Jungbauer and Beck (2002) tested 2,4-D in a yeast based system. The concentrations specified were not identified nor were data provided. This study was not cited as OSRI by the submitter due to the limitations of yeast-based assays.

- Lee *et al.* (2006) used a yeast- based two-hybrid system. 2,4-D was reported to be positive in this system with binding over the range 2.09 X 10^{-4} to 5.42 X 10^{-6} M. This study was not cited as OSRI by the submitter due to the limitations of yeast-based assays.

- Lin and Garry (2000) tested 2,4-D in an MCF-7 cell proliferation assay. Technical grade 2,4-D was positive in the assay, but the reagent grade was negative. A cell proliferation assay does not measure receptor binding and may be affected by factors other than binding with the receptor. It may therefore be useful ancillary information, but cannot satisfy the test order requirement.

- Kojima *et al.*, (2004) tested 2,4-D (> 95% purity) in CHO-K1 cell transcriptional activation assay and reported negative results. No data were provided to substantiate this result. EPA would reconsider the adequacy of this study if supporting data were submitted.

- Lemaire *et al.*, (2006) tested 49 pesticides in HeLa cells transfected with ERα and ERβ. Substances were tested first at 10 μM. Positive substances were retested to determine a dose response curve and calculate an EC50. 2,4-D was reported negative with a percent activity in ERα and ERβ of 8.4 ± 1.4 and 10.2 ± 2.1 which was not statistically different from the solvent DMSO (9.3 ± 1.3 and 10.8 ± 1.6). There are two issues with this study: first, testing was only conducted at a single concentration, which is inadequate because the results are more susceptible to false positive and false negative responses; and it does not meet Guideline 890.1300 which recommends a full concentration response curve.

- Nishihara *et al.*, (2000) conducted a yeast two hybrid assay. It was tested up to 10^{-3}M and found to be negative. The limitations of yeast based assays were discussed in the paper and recognized by the submitter; the submitter did not consider the study to be OSRI. Nishihara notes that his test system may give false negative results for various reasons including the following: 1) some act *via* receptors other than ER; 2) some involve a pathway other than *via* receptor-mediated gene expression; 3) some act as antagonists; 4) some act after being metabolized by animal cells; 5) some have inhibitory activity against the galactosidase assay and biocidal activity against the yeast cell; and 6) some cannot be transported into the cell, resulting in cellular concentrations below the sensitivity level.

Table 5. Evaluation of Data Submitted in Relation to the Estrogen Receptor Transcriptional Activation Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1300 - Estrogen Receptor Transcriptional Activation Assay (Human Cell Line HeLa-9903)

- Orton *et al.,* (2009) conducted a yeast estrogen screen on 2,4-D. It was reported negative however, no data were provided. In addition, the assay is subject to the limitations of yeast based assays described above.

- Petit *et al.,* (1997) tested 2,4-D in three different *in vitro* assays. It was tested in a yeast transactivation assay over a range of 10^{-8} to 10^{-4} M in which the yeast was transfected with rainbow trout ERα using a β-galactosidase reporter. Similarly a competitive binding assay was run using yeast cells and rainbow trout ERα. It was also tested for its ability to induce vitellogenin mRNA in a primary rainbow trout hepatocyte culture. The assay results were reported as follows: β-Gal expression in the TA assay = 17.95%, 8.0% Vg induction, and fold induction needed to displace 50% of bound E2 > 10,000. All results were described as negative. As noted, yeast based systems are not the equivalent to mammalian systems. This may be ancillary information, but it does not satisfy the test order.

- Soto *et al.,* (1995) conducted an E-Screen (MCF-7 cell proliferation assay) on 2.4-D. Negative results were reported but not data were provided to substantiate this conclusion. In addition, the cell proliferation assay is not a transcriptional activation assay recommended by 890.1300 and is not equivalent to a mammalian transcriptional activation assay because cell proliferation can have other causes than binding to the estrogen receptor. Consequently, this assay would only provide ancillary information even if data were provided.

- Vonier *et al.,* (1996) tested 2,4-D in a competitive binding assay using ER derived from alligator oviducts. 2,4-D was reported to be a non-binder, but no data were shown to substantiate this conclusion and the purity and range over which 2,4-D was tested were not specified. This study is not a transcriptional activation study and does not meet Guideline 890.1300.

- Xie *et al.,* (2005). This study evaluated vitellogenin induction in immature trout exposed to 0.00164, 0.0164, 0.164 and 1.64 mg/L of 2,4-D. The results showed a concentration-response relationship with the top two concentrations being significantly different from controls. While this study shows evidence of estrogenic activity, it is not a transcriptional activation assay and is not as specific for estrogen receptor mediated activity. A positive response in a vitellogenin assay could also reflect induction of aromatase. Therefore this assay does not satisfy the requirements of the test order.

Table 5. Evaluation of Data Submitted in Relation to the Estrogen Receptor Transcriptional Activation Assay	
Chemical: 2,4-D	**PC Code: 031001**
890.1300 - Estrogen Receptor Transcriptional Activation Assay (Human Cell Line HeLa-9903)	
• The PETA submission provided a brief review of a few human studies that addressed potential reproductive and developmental toxicity of 2,4-D based on the exposure of Vietnam veterans to Agent Orange. The conclusion was that the veterans who had greater potential exposure to Agent Orange did not have an increased risk of fathering babies with major structural birth defects. In fact, even for pesticide applicators and the general population in an agricultural region of Minnesota, a more detailed cross-sectional analysis of this area showed no statistically significant correlation between 2,4-D use and excess adverse birth effects. The Agency believes that overall, prospective cohort studies still need to be conducted to confirm or test the hypothesis generated by these studies. In addition, additional detail is needed on exposures of the subjects to drugs and chemicals which may have an adverse effect on the male reproductive system that can contribute confounding effects in the outcome and interpretation of these data. While these data provide additional information that upon further investigation may contribute to hazard characterization, they do not provide confirmed or confident linkages between human exposure to 2,4-D and the potential for transactivation of the estrogen receptor. • None of the cited Part 158 studies measure activation of estrogen-receptor-controlled DNA transcription, which is the information that would be obtained by the Tier 1 ER Transcriptional Activation Assay.	
4. Conclusion: Based on the deficiencies and outstanding questions listed above, the data cited as OSRI did not satisfy the requirement for the Estrogen Receptor Transcriptional Activation Assay using Guideline 890.1300. EPA would reconsider this conclusion if supporting data were submitted for Kojima *et al.* (2004).	

[1] -- = not measured; X = indicates the endpoint was measured in the assay but does not indicate whether or not it satisfies the data requirement of the test order. See section 3 below for a detailed explanation; N/A = not applicable

Table 6. Evaluation of Data Submitted in Relation to the Fish Short-Term Reproduction Assay

Chemical: 2,4-D	**PC Code: 031001**

890.1350 - Fish Short-Term Reproduction

1. EDSP Assay Endpoints[1]

Study Type / Literature Citation	**MRID No.**	**Reproductive Behavior and Secondary Sex Characteristics**						
		Fecundity	**Fertility**	**Vitellogenin**	**Sex Steroid Concentration**	**Secondary Sex Characteristics**	**Gonado-Somatic Index**	**Gonadal Histopathology**
N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

2. Summary of Study Findings:

Study Type / Literature Citation	**MRID No.**	**Findings**
N/A	N/A	N/A

3. Agency's Evaluation of the OSRI: N/A

4. Conclusion:

The Test Order Recipient has agreed to conduct this assay.

[1] -- = not measured; X = indicates the endpoint was measured in the assay but does not indicate whether or not it satisfies the data requirement of the test order. See section 3 below for a detailed explanation; N/A = not applicable

Table 7. Evaluation of Data Submitted in Relation to the Hershberger Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1400 - Hershberger Assay

1. EDSP Assay Endpoints[1]

Study Type / Literature Citation	MRID No.	Tissue Weight				
		Ventral Prostate	Seminal Vesicle	LABC Muscle	Cowper's Glands	Glans Penis
ToxCast	N/A	--	--	--	--	--
Fang *et al.*, (2003)	48074110	--	--	--	--	--
Kim *et al.*, (2005)	N/A	--	--	--	--	--
Kojima *et al.*, (2004)	48033008	--	--	--	--	--
Orton *et al.*, (2009)	48041727	--	--	--	--	--
Extended 1-Generation Reproduction - Rat	47972101	--	--	--	--	--
2-Generation Reproduction - Rat	00150557	--	--	--	--	--
Developmental Toxicity – 2,4-D Acid - Rat	00130407	--	--	--	--	--
Developmental Toxicity – 2,4-D Salts & Esters – Rat [Charles *et al.*, (2001)]	45761204	--	--	--	--	--
Developmental Toxicity –2,4-D Acid Rabbit	41747601	--	--	--	--	--
Developmental Toxicity –2,4-D Salts& Esters – Rabbit [Charles *et al.*, (2001)]	45761204	--	--	--	--	--
Chronic Toxicity/Carcinogenicity – Rat	43612001	--	--	--	--	--
Carcinogenicity – Male Mouse	43879801	--	--	--	--	--
1981: Subchronic Oral toxicity - Rat	00102451	--	--	--	--	--
1991: Subchronic Oral Toxicity- 2,4-D Acid - Rat	41991501	--	--	--	--	--

Table 7. Evaluation of Data Submitted in Relation to the Hershberger Assay						
Chemical: 2,4-D		**PC Code: 031001**				
890.1400 - Hershberger Assay						
Subchronic Oral Toxicity – 2,4-D Salts & Esters – Rat [Charles *et al.*, (1996)]	45761213	--	--	--	--	--
Subchronic Oral Toxicity -Mouse	41991502	--	--	--	--	--
Subchronic Inhalation Toxicity- Rat	47398701	--	--	--	--	--
Epidemiology Studies (PETA OSRI)	N/A	--	--	--	--	--

2. Summary of Study Findings:

Study Type / Literature Citation	MRID No.	Findings
ToxCast	N/A	See discussion below in Section 3.
Fang *et al.*, (2003)	48074110	See discussion below in Section 3.
Kim *et al.*, (2005)	N/A	See discussion below in Section 3.
Kojima *et al.*, (2004)	48033008	See discussion below in Section 3.
Orton *et al.*, (2009)	48041727	See discussion below in Section 3.
Extended 1-Generation Reproduction - Rat	47972101	In the P1 animals, male and female mating, conception, fertility, and gestation indices were comparable among the groups, and post-implantation loss was comparable among the groups. Both the time to mating and gestation length was comparable among the groups. The decreases observed in the weights of the testes, epididymides, seminal vesicles and the prostate at the mid and high dose groups were not attributed to treatment since the values in the concurrent control were outside of the laboratory historical control range. There were no treatment-related effects on sperm motility or progressive motility, no differences in testicular spermatid and epididymal sperm counts, and no differences in the proportion of abnormal sperm.

Table 7. Evaluation of Data Submitted in Relation to the Hershberger Assay		
Chemical: 2,4-D		**PC Code: 031001**
890.1400 - Hershberger Assay		
		In the F1 Offspring (PND 70), there were no treatment-related effects on the numbers of live or dead F1 pups born/litter or on pup survival or sex ratio. There were no significant, treatment-related differences in absolute or relative anogenital distance in male pups and no differences in nipple/areolae retention between control and high-dose groups in male pups. Male pups at the high dose displayed a slight delay in preputial separation (1.6 days), which was accompanied by a very slight reduction in body weight compared to the control (↓2.1 grams; 99% of control). No histopathological lesions were seen in the testes, epididymides, seminal vesicle and the prostate glands. Although statistical significance was not attained, treatment-related changes observed in the absolute weights of the prostate (↓6%) and epididymides in males at the high dose. In the F1 Offspring (PND139), no treatment-related effects were seen on sperm motility or progressive motility, no differences in testicular spermatid and epididymal sperm counts, and no differences in the proportion of abnormal sperms. No treatment-related histopathological lesions were seen in the testes, epididymides, seminal vesicles, prostate, ovaries, oviducts, uterus, vagina, cervix, mammary, thyroid, adrenal and pituitary glands of PND 139 animals.
2-Generation Reproduction - Rat	00150557	In the F0 generation, no apparent adverse effect was observed on fertility. Pre-coital intervals were comparable among the groups. The duration of gestation was increased (0.6 days) at the high-dose of F0 rats producing the F1b pups. The gestation survival index was comparable among the groups for the F1a pups but was significantly decreased for the F1b litters. There was a significant decrease in the number of F1a female fetuses at the high-dose. The number of F1b pups born dead/dying by day 1 was significantly increased at the high-dose. Fl a litter size was slightly lower at the high-dose, but F1b litter size was significantly lower than the control. Fla pup viability was comparable throughout weaning, but the Flb pup viability was significantly lower throughout the weaning period.

Table 7. Evaluation of Data Submitted in Relation to the Hershberger Assay		
Chemical: 2,4-D		**PC Code: 031001**
890.1400 - Hershberger Assay		
		There was a significant decrease in Flb pup survival to lactation day 4 at the high-dose level as well as survival to lactation day 28. In the F1 generation, no apparent adverse effect was observed on fertility at either dose level. Pre-coital intervals and gestation lengths were comparable among the groups. The gestation survival index and the viability index were comparable among the groups for both the F2a and F2b litters. Litter size, body weights, and the sex ratio were comparable among the groups in both the F2a and F2b litters. No treatment-related changes in absolute or relative testes weights or any treatment-related histopathological lesions were seen in the testes of offspring of any generation.
Developmental Toxicity - 2,4-D Acid - Rat	00130407	No treatment-related changes were seen in pregnancy rate, number of corpora lutea, mean number of implantations/litter, post-implantation loss, early or late resorptions, number of live fetuses/litter, fetal sex ratio, or soft tissue abnormalities at any dose.
Developmental Toxicity – 2,4-D Salts & Esters – Rat [Charles *et al.*, (2001)]	45761204	No treatment-related changes were seen in pregnancy rate, number of corpora lutea, mean number of implantations/litter, post-implantation loss, early or late resorptions, number of live fetuses/litter, fetal sex ratio, or soft tissue abnormalities for 2,4-D acid or any of its amine salts (2,4-D DMA; 2.4-D DEA; 2.4-D IPA; 2.4-D TIPA) or esters (2.4-D BEE, 2.4-D EHE and 2,4-DIPE).
Developmental Toxicity –2,4-D Acid Rabbit	41747601	No treatment-related changes were seen in pregnancy rate, number of corpora lutea, mean number of implantations/litter, post-implantation loss, early or late resorptions, number of live fetuses/litter, fetal sex ratio, or soft tissue abnormalities at any dose.
Developmental Toxicity – 2,4-D Salts& Esters – Rabbit [Charles *et al.*, (2001)]	45761204	No treatment-related changes were seen in pregnancy rate, number of corpora lutea, mean number of implantations/litter, post-implantation loss, early or late resorptions, number of live fetuses/litter, fetal sex ratio, or soft tissue abnormalities for 2,4-D acid or any of its amine salts (2,4-D DMA; 2.4-D DEA; 2.4-D IPA; 2.4-D TIPA) or esters (2.4-D BEE and 2.4-D EHE).

Table 7. Evaluation of Data Submitted in Relation to the Hershberger Assay		
Chemical: 2,4-D		**PC Code: 031001**
890.1400 - Hershberger Assay		
Chronic Toxicity/Carcinogenicity – Rat	43612001	Treatment-related changes seen in the testes were: • Absolute weights were significantly ($p<0.05$) decreased (15%) at the 150 mg/kg/day at the interim (12-month) sacrifice. • Absolute (52%) and relative (49%) testes weights were decreased at 150 mg/kg/day at the terminal (24-month) sacrifice. • Atrophy of the testes was seen in 2/50 at 150 mg/kg/day compared to 0/50 in the controls.
Carcinogenicity – Male Mouse	43879801	Male reproductive organ weights were not evaluated. No treatment-related histopathological lesions were seen in the testes, epididymides, seminal vesicles and prostate glands.
1981: Subchronic Oral toxicity - Rat	00102451	No treatment-related changes in absolute or relative testes weight were seen. No treatment-related histopathological lesions were seen in the testes, epididymides, accessory sex glands and prostate glands.
1991: Subchronic Oral Toxicity- Acid - Rat	41991501	Absolute and relative testes weights were significantly ($p <0.05$) increased (51% absolute and 67% relative) at the high dose. Atrophy of the testes was seen in 8/10 at the high dose compared to 0/10 in the controls.
Subchronic Oral Toxicity – Salts & Esters – Rat [Charles *et al.*, (1996)]	45761213	Relative testes weights were significantly ($p <0.05$) decreased in at the high dose for all three compounds. No treatment-related histopathological lesions were seen in the testes and epididymides.
Subchronic Oral Toxicity –Mouse	41991502	No treatment-related changes in absolute or relative weights of the testes or histopathological lesions were seen in the testes and epididymides.
Subchronic Inhalation Toxicity- Rat	47398701	No treatment-related changes in absolute or relative weights of the testes, nor histopathological lesions in the testes, epididymides, seminal vesicles and prostate glands.
Epidemiology Studies (PETA OSRI)	N/A	See discussion in Section 3 below.

Table 7. Evaluation of Data Submitted in Relation to the Hershberger Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1400 - Hershberger Assay

3. Agency's Evaluation of the OSRI:

The submission provided by the Test Order Recipient stated that "The Hershberger assay primary objective is to assess potential androgenicity or anti androgenicity. *In vitro* ToxCast® studies conducted under the auspices of the US EPA and cited as OSRI showed no evidence of 2,4-D interaction with the androgen receptor. Review of published *in vitro* data, reviewed in Appendix III, confirms the general lack of androgen receptor binding or transactivation in several test systems. As discussed in the text and summarized in the matrix for the Hershberger assay in Appendix II, review of the reproductive toxicity and subchronic and chronic toxicity regulatory toxicity data for 2,4-D cited as OSRI, as well as other supplementary studies, do not show any consistent patterns of effects related to androgenicity at any dose, or to anti-androgenicity at doses not markedly exceeding the KMD, or renal excretion saturation threshold for 2,4-D. 2,4-D was evaluated in two reproductive toxicity studies including an F1-extended one generation study which incorporated multiple endocrine related endpoints. These evaluations overlapped the exposure time frame for the Hershberger assay, and arguably the reproductive study design tests a more sensitive time frame, due to the perinatal exposure in this study paradigm. Parameters evaluated in this study included many sensitive to anti-androgenicity or androgenicity, including balano-preputial separation, anogenital distance, quantitative F1 nipple retention, spermiology, organ weights and detailed histopathological evaluations of male reproductive organs. No exposure-related effects were seen for those parameters (Marty *et al.,* 2010). Decreased testicular weights and testicular atrophy have been reported in a subchronic toxicity study at doses causing extensive systemic toxicity and far exceeding the KMD (Schulze, 1991a)."

"One published study (Kim *et al.,* 2002, available in Korean only) reports organ weight changes in a Hershberger assay suggesting androgenicity at a single high dose level likely to exceed the KMD. This study cannot be evaluated because too few data are presented; however the findings are inconsistent with the findings in the regulatory toxicity data base. Based on the extensive data cited as OSRI, the 2,4-D Task Force requests a waiver from a Hershberger assay."

The PETA comments submitted to the Agency in response to the EDSP screening order issued for 2,4-D stated that "In summary, 2,4-D is an extremely well-studied chemical. Its endocrine disrupting potential has already been addressed in mammalian subchronic, reproductive and developmental studies as well as in numerous *in vitro* assays, in chronic studies in birds, fish and reptiles and in epidemiologic studies. Further, a study designed in consultation with the EPA to definitively address remaining endocrine-sensitive endpoints is nearing completion. There is no need for further testing under the EDSP."

Table 7. Evaluation of Data Submitted in Relation to the Hershberger Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1400 - Hershberger Assay

On review of the OSRI submitted, the Agency noted the following observations with regard to the androgen pathway:

- Although the cited ToxCast data may be appropriate for use in priority setting, ToxCast *in vitro* assays cannot at this time be considered acceptable alternatives to the EDSP Tier 1 *in vivo* assays. Thus this information is not considered sufficient to satisfy the Test Order requirement for the Hershberger Assay using Guideline 890.1400 (Kavlock and Zenick, 2010).

- Fang *et al.*, (2003) reported data for each competitor and R1881 (standard AR ligand) standard curve were plotted as [^{3}H]R1881 bound (relative to the standard) vs. molar concentrations. 2,4-D was adequately tested at concentrations from 4.28×10^{-9} to 4.28×10^{-4} M. All assays were run in duplicate with at least two replications. Appropriate reference chemicals were used in the study. The IC_{50} (50% inhibition of R1881 standard) values were reported and the relative binding affinities (RBA) were calculated for each chemical. Based on the RBA values, the chemicals were classified as strong binders, moderate binders, weak binders or inactive (non-binders). 2,4-D was classified in this study as a non-binder. The authors compared the recombinant AR binding data generated in this study to a published data set that was generated using the rat ventral prostate cytosol AR method similar to the one used in the EDSP Tier 1 assay. There were 20 chemicals in common between the two data sets. The results from the two methods were generally comparable with the recombinant AR method demonstrating adequate sensitivity to identify strong, moderate and weak androgen receptor binders. Radiolabeled [^{3}H]-R1881 is used to measure competitive AR binding (similar to the Tier 1 assay). The submissions in response to the test order did not provide a clear rationale that explains how *in vitro* data could provide equivalent information to that which would be obtained from the Hershberger Assay.

- Kim *et al.*, (2005) studied the ability of 2,4-D and its metabolite to increase the proliferation of 22Rv1 human prostate cancer cells. Negative results were reported. Trancriptional activation studies were conducted with various cell lines. All were reported negative, except 2,4-D was reported to increase the response with co-administered DHT. Due to the limitations of these studies, the submitter did not claim them as OSRI.

- Kojima *et al.*, (2004) conducted a transcriptional activation assay and tested a narrower range of concentrations than recommended by OPPTS Guideline 890.1250. The submissions in response to the test order did not provide a clear rationale that explains how *in vitro* data could provide equivalent information to that which would be obtained from the Hershberger Assay.

Table 7. Evaluation of Data Submitted in Relation to the Hershberger Assay	
Chemical: 2,4-D	**PC Code: 031001**
890.1400 - Hershberger Assay	

- Orton *et al.*, (2009) conducted the yeast androgen screen with 2,4-D. 2,4-D was reported to be negative. No data were reported to substantiate the negative result, which is particularly significant, as false negatives can result in the yeast assay as some chemicals do not cross the yeast cell wall. Nor did the submissions in response to the test order provide any rationale that explains how *in vitro* data could provide equivalent information to that which would be obtained from the Hershberger Assay.

<u>In the Extended One-Generation Reproduction Study:</u>

- There were no significant effects on any of the reproductive indices in the P1 rats at any dose level. Male mating, conception, fertility, and gestation indices, the percent post-implantation loss, and the sex ratio were comparable among the groups.
- There were no significant treatment related effects on sperm counts, sperm morphology or sperm motility in P1 and F1 offspring.
- There were no significant treatment-related differences in anogenital distance (AGD) in either sex in the F1.
- There was a slight delay (1.6 days) in preputial separation at the high dose (40 mg/kg/day) with a slight (2.1 g; 99% of control) in body weight. This delay was not thought to be biologically relevant.
- External male genitalia were normal at birth and there was no difference in nipple/areolae retention in male pups.
- No treatment-related effects were seen in the absolute or relative weights of the androgen sensitive organs (testes, epididymides, seminal vesicles and prostate) in P1 or F1 offspring.
- No treatment-related histopathological lesions were seen in any of the androgen sensitive organs (testes, epididymides, seminal vesicles and prostate) of P1 or F1 (PND 70 and 139) offspring.
- No treatment-related effects were seen in the absolute or relative weights of the pituitary nor were there any histopathological lesions of the pituitary glands in P1 or F1 (PND 70 and 139) offspring.

<u>In the Chronic Toxicity/Carcinogenicity Study in the rat:</u>

- Testes weights were significantly decreased in rats at the high dose at both the interim (absolute weights) and terminal (absolute and relative weights) sacrifices, and
- Atrophy of the testes was seen in 2/50 at 150 mg/kg/day compared to 0/50 in the controls.

<u>In the Subchronic Toxicity Study in the rat:</u>

- Absolute and relative testes weights were significantly increased at the high dose, and
- Atrophy of the testes was seen in 8/10 at the high dose compared to 0/10 in the controls.

Table 7. Evaluation of Data Submitted in Relation to the Hershberger Assay	
Chemical: 2,4-D	**PC Code: 031001**
890.1400 - Hershberger Assay	
• The PETA submission provided a brief review of a few human studies that addressed potential reproductive and developmental toxicity of 2,4-D based on the exposure of Vietnam veterans to Agent Orange. The conclusion was that the veterans who had greater potential exposure to Agent Orange did not have an increased risk of fathering babies with major structural birth defects. In fact, even for pesticide applicators and the general population in an agricultural region of Minnesota, a more detailed cross-sectional analysis of this area showed no statistically significant correlation between 2,4-D use and excess adverse birth effects. The Agency believes that overall, prospective cohort studies still need to be conducted to confirm or test the hypothesis generated by these studies. In addition, additional detail is needed on exposures of the subjects to drugs and chemicals which may have an adverse effect on the male reproductive system that can contribute confounding effects in the outcome and interpretation of these data. While these data provide additional information that upon further investigation may contribute to hazard characterization, they do not provide confirmed or confident linkages between human exposure to 2,4-D and interaction with the androgen pathway.	
4. Conclusion: The requirement for the **Hershberger Assay (890.1400) is satisfied** based on the Extended One-Generation Rat Reproduction Study which provided numerous detailed measures of the endocrine system in developing offspring as a consequence of pre-natal and post-natal exposures to 2,4-D.	

[1] -- = not measured; X = indicates the endpoint was measured in the assay but does not indicate whether or not it satisfies the data requirement of the test order. See section 3 below for a detailed explanation; N/A = not applicable

Table 8. Evaluation of Data Submitted in Relation to the Female Pubertal Assay										
Chemical: 2,4-D					**PC Code: 031001**					
890.1450 - Female Pubertal Assay (Rat)										
1. EDSP Assay Endpoints[1]				**Organ Weights**						
Study Type / Literature Citation	**MRID No.**	**Growth**	**Age and Weight at VO**	**Uterus**	**Ovaries**	**Thyroid**	**Liver**	**Kidneys**	**Pituitary**	**Adrenals**
Evangelista de Duffard *et al.* (1990)	N/A	--	--	--	--	--	--	--	--	--
Petit *et al.* (1997)	N/A	--	--	--	--	--	--	--	--	--
Xie *et al.* (2005)	N/A	--	--	--	--	--	--	--	--	--
Extended 1-Generation Reproduction - Rat	47972101	x	x	x	x	x	x	x	x	x
2-Generation Reproduction - Rat	00150557	x	--	--	--	--	x	x	--	--
Developmental Toxicity- Acid – 2,4-D Rat	00130407	x	--	--	--	--	--	--	--	--
Developmental Toxicity- 2,4-D Salt & Esters – Rat [Charles *et al.*, (2001)]	45761204	x	--	--	--	--	--	--	--	--
Developmental Toxicity- 2,4-D Acid - Rabbit	41747601	x	--	--	--	--	--	--	--	--
Developmental Toxicity –2,4-D Salt & Esters - Rabbit [Charles *et al.*, (2001)]	45761204	x	--	--	--	--	--	--	--	--

Table 8. Evaluation of Data Submitted in Relation to the Female Pubertal Assay										
Chemical: 2,4-D					**PC Code: 031001**					
890.1450 - Female Pubertal Assay (Rat)										
Chronic Toxicity /Carcinogenicity – Rat	43612001	x	--	--	x	x	x	x	--	x
Carcinogenicity – Female Mouse	43879801	x	--	--	x	--	x	x	--	--
1981: Subchronic Oral toxicity – Rat	00102451	x	--	--	--	--	x	x	--	--
1991: Subchronic Oral Toxicity- Acid - 2,4-D Rat	41991501	x	--	--	x	x	x	x	x	x
Subchronic Oral Toxicity – 2,4-D Salts & Esters – Rat [Charles *et al.*, (1996)]	45761213	x	--	--	x	x	x	x	x	x
Subchronic Oral Toxicity –Mouse	41991502	x	--	--	x	x	x	x	x	x
Subchronic Inhalation Toxicity- Rat	47398701	x	--	x	x	--	x	x	--	x
Avian Reproduction Study with Bobwhite Quail (*Colinus virginianus*)	45336401	x	--	--	--	--	--	--	--	--
Epidemiology Studies (PETA OSRI)	N/A	--	--	--	--	--	--	--	--	--

Table 8. Evaluation of Data Submitted in Relation to the Female Pubertal Assay									
Chemical: 2,4-D					**PC Code: 031001**				
890.1450 - Female Pubertal Assay (Rat)									
Study Type / Literature Citation	**MRID No.**	**Clinical Chemistry, Hormone, Pathology, Cyclicity [1]**							
		Histopathology				**Blood Chemistry**	**Hormones**		**Estrous Cyclicity (Age, Length & % of animals Cycling)**
		Uterus	**Ovary**	**Thyroid**	**Kidney**		**T4**	**TSH**	
Evangelista de Duffard *et al.*, (1990)	N/A	--	--	--	--	--	--	--	--
Petit *et al.*, (1997)	N/A	--	--	--	--	--	--	--	--
Xie *et al.*, (2005)	N/A	--	--	--	--	--	--	--	--
Extended 1-Generation Reproduction – Rat	47972101	x	x	x	x	x	x	x	x
2-Generation Reproduction - Rat	00150557	--	x	--	x	--	--	--	--
Developmental Toxicity- 2,4-D Acid – Rat	00130407	--	--	--	--	--	--	--	--
Developmental Toxicity-2,4-D Salt & Esters – Rat [Charles *et al.*, (2001)]	45761204	--	--	--	--	--	--	--	--
Developmental Toxicity- Acid -Rabbit	41747601	--	--	--	--	--	--	--	--
Developmental Toxicity–2,4-D Salt & Esters - Rabbit Charles *et al.*, (2001)	45761204	--	--	--	--	--	--	--	--

Table 8. Evaluation of Data Submitted in Relation to the Female Pubertal Assay									
Chemical: 2,4-D					**PC Code: 031001**				
890.1450 - Female Pubertal Assay (Rat)									
Chronic toxicity /Carcinogenicity – Rat	43612001	x	x	x	x	x	x	--	--
Carcinogenicity – Female Mouse	43879801	x	x	x	--	--	--	--	--
1981: Subchronic Oral toxicity - Rat	00102451	x	x	x	x	x	x	--	--
1991: Subchronic Oral Toxicity- 2,4-D Acid – Rat	41991501	x	x	x	x	--	--	--	--
Subchronic Oral Toxicity – 2,4-D Salts & Esters – Rat [Charles *et al.*, (1996)]	45761213	x	x	x	x	x	x	--	--
Subchronic Oral Toxicity –Mouse	41991502	x	x	x	x	x	x	--	--
Subchronic Inhalation Toxicity- Rat	47398701	x	x	x	x	x	--	--	--
Avian Reproduction Study with Bobwhite Quail (*Colinus virginianus*)	45336401	--	--	--	--	--	--	--	--
Epidemiology Studies (PETA OSRI)	N/A	--	--	--	--	--	--	--	--
2. Summary of Study Findings:									
Study Type / Literature Citation	**MRID No.**	**Findings**							
Evangelista de Duffard *et al.* (1990)	N/A	See Section IV of this report.							

Table 8. Evaluation of Data Submitted in Relation to the Female Pubertal Assay		
Chemical: 2,4-D		**PC Code: 031001**
890.1450 - Female Pubertal Assay (Rat)		
Petit *et al.* (1997)	N/A	2,4-D was tested in three different *in vitro* assays; a yeast transactivation assay over a range of 10^{-8} to 10^{-4} M in which the yeast was transfected with rainbow trout ERα using a β-galactosidase reporter; a competitive binding assay using yeast cells and rainbow trout ERα; and the induction of vitellogenin mRNA in a primary rainbow trout hepatocyte culture. All results were described as negative. However, it is noted that yeast based systems are not the equivalent of mammalian systems.
Xie *et al.* (2005)	N/A	This study evaluated vitellogenin induction in immature trout exposed to 2,4-D. Although positive findings were reported, this study suffered from significant endpoint response variability and the findings were inconsistent with *in vitro* studies suggesting a lack of ER binding or activation in trout as reported by Petit *et al* 1997 as discussed above.
Extended 1-Generation Reproduction – Rat	47972101	In the P1 animals, female mating, conception, fertility, and gestation indices were comparable among the groups, and post-implantation loss was comparable among the groups. Both the time to mating and gestation length were comparable among the groups. There were no alterations in estrous cycle pattern in females at the high dose, and no significant difference in mean estrous cycle length in P1 females at any dose level compared to the control. There was a non-statistically significant increase in uterine weights (↑17%, both absolute and relative) at the high dose. In the GD 17 females, reproductive indices and the numbers of corpora lutea and implantations were comparable among the groups. There was a slight increase in resorptions at the high dose (0.9 vs 1.5), although there was wide variability (standard deviations exceed the means). There was a slight increase in post-implantation loss at the high dose (9.2 vs 5.5). There were no statistically significant, treatment-related differences in serum T3, T4, or TSH levels. Both the mid and high dose group females displayed an increase in thyroid weight (↑9%), but there was no dose-response. Smaller follicles (colloid resorptions) were seen in 3/12 females compared 0/12 in the controls. In the F1 offspring (PND 70), there was no significant, treatment-related difference in absolute or relative anogenital distance in either sex and no differences in nipple/areolae retention between control and high-dose groups in either sex. The age at vaginal opening was comparable among the groups of F1 females. In the PND 4 culled pups, there were no treatment-related differences in serum T3, T4, or TSH levels. T4 was reduced to a similar extent in females at the mid (↓14%-15%) and high

Table 8. Evaluation of Data Submitted in Relation to the Female Pubertal Assay		
Chemical: 2,4-D		**PC Code: 031001**
890.1450 - Female Pubertal Assay (Rat)		
		(↓12%-14%) dose levels, and there was an increase in TSH (↑19%) level at the high dose. In the PND 22 weanling, there was a non-statistically significant reduction (↓20%) in T4 at the high dose. In the PND 62-64 offspring, there was an increase in TSH level (↑24%) at the high dose; none of these differences reached statistical significance. In the F1 Offspring (PND139), no treatment-related differences were observed in mean estrous cycle length at any dose level compared to the control. There were no significant, treatment-related effects on the numbers of small follicles, growing follicles, or total follicles. Uterine weights were increased at the mid (↑10% absolute and ↑10% relative) and high (↑10% absolute and ↑11% relative) dose groups. Ovarian follicle counts were comparable between the control and high dose females. Absolute (↓9%) and relative (↓8%) pituitary gland weights were significantly lower in males at the high dose and the absolute (↓9%) and relative (↓10%) pituitary gland weights were non-significantly lower in females at the high dose. There was no associated histopathology in the pituitary glands. No treatment-related histopathological lesions were seen in the ovaries, oviducts, uterus, vagina, cervix, mammary, thyroid, adrenal and pituitary glands of PND 139 animals.
2-Generation Reproduction - Rat	00150557	In the F0 generation, no apparent adverse effect was observed on fertility. Pre-coital intervals were comparable among the groups. The duration of gestation was increased (0.6 days) at the high-dose of F0 rats producing the F1b pups. The gestation survival index was comparable among the groups for the F1a pups but was significantly decreased for the F1b litters. There was a significant decrease in the number of F1a female fetuses at the high-dose. The number of F1b pups born dead/dying by day 1 was significantly increased at the high-dose. Fla litter size was slightly lower at the high-dose, but F1b litter size was significantly lower than the control. Fla pup viability was comparable throughout weaning, but the Flb pup viability was significantly lower throughout the weaning period. There was a significant decrease in Flb pup survival to lactation day 4 at the high-dose level as well as survival to lactation day 28. In the F1 generation, no apparent adverse effect was observed on fertility at either dose level. Pre-coital intervals and gestation lengths were comparable among the groups. The gestation survival index and the viability index were comparable among the groups for both the F2a and F2b litters. Litter size, body weights, and the sex ratio were comparable among the groups in both the F2a and F2b litters. No treatment-related changes in absolute or relative testes weights nor were there any treatment-related histopathological lesions seen in the ovaries of offspring of any

Table 8. Evaluation of Data Submitted in Relation to the Female Pubertal Assay		
Chemical: 2,4-D		**PC Code: 031001**
890.1450 - Female Pubertal Assay (Rat)		
		generation.
Developmental Toxicity- Acid –2,4-D Rat	00130407	No treatment-related changes were seen in pregnancy rate, number of corpora lutea, mean number of implantations/litter, post-implantation loss, early or late resorptions, number of live fetuses/litter, fetal sex ratio, or soft tissue abnormalities at any dose.
Developmental Toxicity- 2,4-D Salt & Esters – Rat [Charles *et al.*, (2001)]	45761204	No treatment-related changes were seen in pregnancy rate, number of corpora lutea, mean number of implantations/litter, post-implantation loss, early or late resorptions, number of live fetuses/litter, fetal sex ratio, or soft tissue abnormalities for 2,4-D acid or any of its amine salts (2,4-D DMA; 2.4-D DEA; 2.4-D IPA; 2.4-D TIPA) or esters (2.4-D BEE, 2.4-D EHE and 2,4-DIPE).
Developmental Toxicity- Acid -2,4-D Rabbit	41747601	No treatment-related changes were seen in pregnancy rate, number of corpora lutea, mean number of implantations/litter, post-implantation loss, early or late resorptions, number of live fetuses/litter, fetal sex ratio, or soft tissue abnormalities at any dose.
Developmental Toxicity–2,4-D Salt & Esters - Rabbit [Charles *et al.*, (2001)]	45761204	No treatment-related changes were seen in pregnancy rate, number of corpora lutea, mean number of implantations/litter, post-implantation loss, early or late resorptions, number of live fetuses/litter, fetal sex ratio, or soft tissue abnormalities for 2,4-D acid or any of its amine salts (2,4-D DMA; 2.4-D DEA; 2.4-D IPA; 2.4-D TIPA) or esters (2.4-D BEE and 2.4-D EHE).
Chronic Toxicity /Carcinogenicity – Rat	43612001	No treatment-related changes were seen in T3 concentrations at any dose. Thyroxin (T4) levels were significantly ($p < 0.05$) decreased in females at all intervals measured (6, 12, 18 and 24 months). Ovarian weight was decreased at the high dose at 12 (9%) and 24 (42%) months and at 75 mg/kg/day (35%) at study termination. Significant ($p < 0.05$) increases in absolute and relative thyroid weights were seen at the mid (75 mg/kg/day) and high (150 mg/kg/day) dose groups at 12 and 24 months. No treatment-related histopathological lesions were seen in the ovaries, uterus, vagina, cervix, mammary, adrenal and pituitary glands. Decreased secretory material in the thyroid follicular epithelial cells was seen in 8 females at 150 mg/kg/day compared to none in the controls.
Carcinogenicity – Female Mouse	43879801	Female reproductive organ weights were not evaluated. No treatment-related histopathological lesions were seen in the uterus, ovaries, oviduct, vagina, cervix, mammary, thyroid, adrenal or pituitary glands.
1981: Subchronic Oral Toxicity - Rat	00102451	Total T4 levels were significantly ($p < 0.05$) decreased 50% and 72% at the 100 and 150 mg/kg/day dose groups, respectively. Organ weights were not evaluated. No treatment-related histopathological lesions were seen in the ovaries, uterus, mammary thyroid, adrenal or pituitary glands.

Table 8. Evaluation of Data Submitted in Relation to the Female Pubertal Assay		
Chemical: 2,4-D		**PC Code: 031001**
890.1450 - Female Pubertal Assay (Rat)		
1991: Subchronic Oral Toxicity- 2,4-D Acid - Rat	41991501	Thyroid hormone levels (T3 and T4) were decreased at the mid (100 mg/kg/day) and high (300 mg/kg/day) dose groups at 6 and 13 weeks. At 6 weeks, T3 levels were decreased (87% not significant) and T4 levels were significantly ($p < 0.05$) decreased (30%). At 13 weeks, T3 levels were significantly ($p < 0.05$) decreased (66%) and T4 levels were significantly ($p < 0.05$) decreased (42%). Absolute and relative thyroid weights were significantly ($p < 0.05$) increased (168% and 126%) at the high dose. Absolute and relative ovarian weights were significantly ($p < 0.05$) increased (58% absolute and 82% relative) at the high dose. Treatment-related histopathological changes were observed primarily in the high-dose group and included: hypertrophy of the zona glomerulosa of the adrenal cortex in 10 of 10 females at the mid and high dose groups and hypertrophy of the thyroid glands in 8 of 10 females at the high dose compared to 3 of 10 control females.
Subchronic Oral Toxicity – 2,4-D Salts & Esters – Rat [Charles *et al.*, (1996)]	45761204	T3 concentrations were significantly ($p < 0.05$) decreased in at the mid (100 mg/kg/day) and high (300 mg/kg/day) dose groups with the 2,4-D DMA salt. T4 concentrations were significantly ($p < 0.05$) decreased at the mid and high dose group for the acid, the 2,4-D DMA salt and 2,4-D, 2-EHE. Relative thyroid weights were significantly ($p < 0.05$) increased in females at the high dose with the acid, the 2,4-D DMA salt and 2,4-D, 2-EHE. No treatment-related changes in absolute or relative weights of the ovaries, adrenals or pituitary glands. Histopathology revealed hypertrophy of the adrenal cortex was seen at the high dose with the acid (10/10); 2,4-D DMA salt (9/10); and 2,4-D 2-EHE (10/10). No treatment-related histopathological lesions were seen in the ovaries, mammary glands, thyroid and pituitary glands.
Subchronic Oral Toxicity -Mouse	41991502	No treatment-related changes in absolute or relative weights of the ovaries, thyroid, adrenals or pituitary glands. No treatment-related histopathological lesions were seen in the uterus, ovaries, thyroid, adrenal or pituitary glands.
Subchronic Inhalation Toxicity- Rat	47398701	No treatment-related changes in absolute or relative weights of the ovaries, uterus and adrenals glands. No treatment-related histopathological lesions were seen in the ovaries, uterus, thyroid, adrenal and pituitary glands.
Avian Reproduction Study with Bobwhite Quail	45336401	Bobwhite quail were exposed to 2,4-D at 0 (control), 147, 382, and 962 mg a.i./kg diet (mean-measured). No treatment-related adverse effects were determined from 2,4-D exposure on reproduction, growth, or survival endpoints for bobwhite quail up to 962 mg a.i./kg diet.

Table 8. Evaluation of Data Submitted in Relation to the Female Pubertal Assay		
Chemical: 2,4-D		**PC Code: 031001**
890.1450 - Female Pubertal Assay (Rat)		
Epidemiology Studies (PETA OSRI)	N/A	See discussion below in Section 3.

3. Agency's Evaluation of the OSRI:

The submission provided by the Test Order Recipient stated that "The Female Pubertal assay is intended to characterize estrogenic or anti-estrogenic effects of the test compound. Androgenic effects may also be observed in this assay. In addition, it evaluates parameters relevant to thyroid, adrenal, renal, hepatic and pituitary structure or function. As noted above 2,4-D showed no consistent evidence of binding to or transactivation of the ER (including ERα or ERβ), of binding to or transactivation of the AR in multiple *in vitro* studies, including data developed for ToxCast® under the auspices of the US EPA. The vast majority of the multiple in vitro studies were negative. There was also no evidence of estrogen agonism or antagonism, and an aromatase inhibition assay of 2,4-D was negative. There were no findings in the regulatory toxicology data base for 2,4-D suggesting an estrogenic or anti-estrogenic effect, even at doses greater than the saturation threshold for renal clearance. As discussed in the text and summarized in the matrix for the Female Pubertal assay in Appendix II, 2,4-D was evaluated in multiple reproductive toxicity studies including an F1-extended one-generation reproductive toxicity study that included multiple endocrine related endpoints (Marty *et al.*, 2010), as well as a conventional 83-4 reproductive toxicity study (Rodwell and Brown, 1985). The exposure timeframes in these evaluations overlap the exposure time frame for the Female Pubertal assay, and arguably the reproductive study design tests a more sensitive time frame, due to the perinatal exposure in this study paradigm. Estrogen sensitive parameters that have been evaluated in the F1-extended one generation study of 2,4-D included: estrous cyclicity assessment in parental females and F1 offspring, time to vaginal opening including assessment of vaginal thread retention, anogenital distance, quantitative ovarian follicle count in F1 females, reproductive organ weight evaluations and detailed histopathology of female reproductive organs. These endpoints are sensitive to estrogenic, androgenic and anti-estrogenic modes of action (MOA) and showed no evidence of exposure-related effects (Marty *et al.*, 2010)."

"High-dose effects were seen in a range-finding study on pup survival and growth at doses exceeding the threshold for saturation of renal clearance (Saghir *et al.*, 2008); however, there is no indication these findings occurred below maternally systemically toxic doses or were related to anti-estrogenicity. A decreased incidence of mammary hyperplasia in the chronic rat study (Jefferies *et al.*, 1995) is most likely due to decreased body weight and fat mass at the high doses tested (greater than the KMD). Decreased ovarian weights were observed in the chronic rat study at doses above the KMD; no correlating histopathological observations were made. One report in the published literature (Evangelista de Duffard *et al.*, 1990) claims 2,4-D disrupted the estrous cycle in rats at a high dose exceeding the KMD; too few data from this study are available to evaluate the basis for this observation. One study reported vitellogenin induction in immature trout exposed to 2,4-

Table 8. Evaluation of Data Submitted in Relation to the Female Pubertal Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1450 - Female Pubertal Assay (Rat)

D (Xie *et al.,* 2005); this study suffered from significant endpoint response variability and the findings were inconsistent with in vitro studies suggesting a lack of ER binding or activation in trout (Petit *et al.,* 1997)."

"The Fish Short-Term Reproduction assay that the 2,4-D Task Force is committing to conduct will further characterize whether 2,4-D has any estrogenic effects in fish. The female pubertal assay requires evaluation of thyroid function and structure. A number of parameters related to thyroid function were measured in the 2,4-D studies conducted for pesticide registration purposes. The F1-extended one-generation study evaluated thyroid hormones (T3, T4 and TSH), weight and histopathology across life stages, and also evaluated auditory startle and brain morphometry data (which have been reported to be sensitive to thyroid disruption) in the DNT component of the study. Exposure related effects in females in the F1-extended one-generation study were limited to non-statistically significant decreases in T3 and T4 and increased TSH, associated with adaptive changes (depleted colloid) in the thyroid follicles of GD 17 females at a dose which clearly exceeded the threshold for saturation of renal clearance. No robust effects on auditory startle or statistically significant brain morphometric changes were seen in the DNT component of the extended one-generation reproductive toxicity study, findings otherwise expected as sequelae to any marked thyroid hormone deficiency. Myelin deposition was also characterized at PND 22 and 60, and no exposure-related changes were observed. Overall, there are no findings suggesting an effect on the thyroid at doses below the saturation threshold for renal clearance (Marty *et al.,* 2010). Decreased T4 has also been reported at doses close to or exceeding the KMD in rat subchronic (Schulze [1991a] and Gorzinski *et al.* [1981a]) and chronic (Jeffries *et al.,* 1995) toxicity studies; thyroid histopathological changes however have been very slight (reduced colloid) or not observed in the subchronic studies, and limited to reduced colloid and parafollicular cell hyperplastic changes in females in the chronic rat study, with no evidence of thyroid neoplasia. Parafollicular cell (clear cell) hyperplasia is not a typical response to decreased T4, which generally leads to follicular cell hyperplasia, and the dose response was not clear for the parafollicular cell finding, making exposure relationship equivocal. Duffard's group in Argentina has reported a number of studies of developmental or adult neurotoxicity of 2,4-D; these studies are high dose evaluations and many suffer from methodological deficiencies which make interpretation and reliability questionable. The findings in these studies were not replicated in the F1-extended one generation study, which as noted, evaluated a number of DNT parameters. NOAELs have been established for the rodent thyroid effects, which are restricted to doses above the KMD. Mechanistic studies indicated displacement of thyroid hormone from thyroid plasma binding proteins as the primary mode of action for high dose specific changes in circulating thyroid hormones. ToxCast® reported no evidence that 2,4-D binds to the thyroid receptor. It should be noted that the potential for thyroid effects will be further evaluated in the amphibian metamorphosis assay (AMA), which the 2,4-D Task Force is committing to conduct. This AMA also provides the redundancy appropriate to a screening battery. Changes in adrenal weights were identified in a subchronic rat study at doses exceeding the KMD; histopathology showed hypertrophy in the zona glomerulosa of the adrenal cortex at the same doses (Schulze, 1991a). The latter tissue is not associated with sex

Table 8. Evaluation of Data Submitted in Relation to the Female Pubertal Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1450 - Female Pubertal Assay (Rat)

hormone synthesis. Similar adrenal weight changes were seen at high doses above the KMD in the rat chronic study, but no corresponding histopathology was found (Jeffries *et al.,* 1995). Pituitary weight changes were seen at high doses in several studies; the direction of the change was inconsistent and there was no correlating histopathology suggesting the findings are not exposure-related."

"The female pubertal assay also requires evaluation of parameters related to renal and liver function; these endpoints have been thoroughly evaluated in the regulatory toxicity studies of 2,4-D, including across life stages in the recentF1-extended one generation study. In *toto*, no clearly exposure-related effects were seen in females on estrogen or androgen sensitive parameters, and no consistent pattern of adverse effects was seen in evaluations of adrenal or pituitary. There is a pattern of relatively slight thyroid toxicity in female rats at doses exceeding the KMD. This effect has been characterized across life stages, the mechanism is understood, and a clear NOAEL determined. Additionally, the potential for thyroid effects will be further evaluated in the amphibian metamorphosis assay, which the2,4-D Task Force is committing to conduct. In view of the extensive evaluations of the potential influence of 2,4-D on the female hormonal system and reproductive toxicity, and the lack of reliable or consistent indicators of any adverse endocrine-mediated effects at doses not exceeding the saturation threshold for renal clearance, we consider the female pubertal assay redundant and wasteful of both animal lives and testing resources. Based on the extensive data cited as OSRI, the 2,4-D Task Force requests a waiver from a Female Pubertal assay."

"The PETA comments submitted to the Agency in response to the EDSP screening order issued for 2,4-D stated that "In summary, 2,4-D is an extremely well-studied chemical. Its endocrine disrupting potential has already been addressed in mammalian subchronic, reproductive and developmental studies as well as in numerous *in vitro* assays, in chronic studies in birds, fish and reptiles and in epidemiologic studies. Further, a study designed in consultation with the EPA to definitively address remaining endocrine-sensitive endpoints is nearing completion. There is no need for further testing under the EDSP."

On review of the OSRI submitted, the Agency made the following observations with regard to the estrogen pathway:

<u>In the Extended One-Generation Reproduction Study:</u>

- Reproductive indices were comparable among the groups [mating, fertility, time to mating, gestation length, pre-implantation loss, number of corpora lutea (GD17 dams), and ovarian follicle counts].
- There were no signs of dystocia in P1 dams and no adverse effects on post-implantation loss, litter size, and pup survival.
- No adverse effects were observed on estrous cycle length or estrous cycle pattern, including a lack of persistent estrus (assessed in all P1 main study and GD 17 females and all F1 Set 3 females).

Table 8. Evaluation of Data Submitted in Relation to the Female Pubertal Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1450 - Female Pubertal Assay (Rat)

- No treatment-related effect was seen on developmental landmarks. AGD and nipple retention was comparable among all groups.
- Age at vaginal opening (all Set 1-3 offspring) was comparable among the groups.
- In all three age groups in which uterine weights were measured [P1 (LD 22), F1 (PND 70 and 139)], increased absolute and relative uterine weights (10%-32%) were observed at the high dose.
- There were no significant, treatment-related changes in other reproductive organ weights in P1 animals or in the PND 22, 70 or 139 offspring.
- No treatment-related histopathological lesions were seen in the ovaries, oviducts, uterus, vagina, cervix and mammary gland of P1 animals or in the PND 70 and 139 offspring.

On review of the OSRI submitted, the Agency made the following observations with regard to the thyroid pathway:

In the Extended One-Generation Reproduction Study:

- In the GD 17 satellite dams, the expected hormone response consistent with a perturbation in thyroid function was seen. These findings correlated with the thyroid alterations observed histologically. There was a comparable increase (↑9%) in thyroid weights at the low and high dose levels but no change in thyroid weight at the mid dose.
- In the F1 PND 4 pups, there were decreases in T3 levels at the low (↓8%) and mid (↓13%) dose groups, but not at the high dose. Decreased T4 levels were seen at the mid (↓15%) and high (↓12%) dose groups but there was no dose response. TSH level was increased (↑19%) at the high dose. Thyroid weight and histopathology data were not collected for this age group.
- In the F1 PND 22 offspring, there was a decrease in T4 (↓20%) only at the high dose. Thyroid weight and histopathology data were not collected for this age group.
- In the F1 PND 62-64 offspring, T3 level was increased (↑11%) only at the high dose. T4 levels were increased (↑19%) both at the mid and high dose groups. There was a dose-dependent increase in TSH levels at the mid (↑11%) and high (↑24%) groups.
- Female offspring (PND 70) displayed decreased absolute thyroid weights at the mid (↓9) and high (↓5) dose groups.
- No treatment-related histopathological lesions were seen in the thyroid glands of P1 or F1 offspring.

Table 8. Evaluation of Data Submitted in Relation to the Female Pubertal Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1450 - Female Pubertal Assay (Rat)

In the Chronic Toxicity/Carcinogenicity Study in the rat:

- No treatment-related changes were seen in T3 concentrations at any dose.
- Thyroxin (T4) levels were significantly ($p < 0.05$) decreased in females at all intervals measured (6, 12, 18 and 24 months).
- Absolute and relative thyroid weights were significantly ($p < 0.05$) increased at the mid (75 mg/kg/day) and high (150 mg/kg/day) dose groups at 12 and 24 months.
- Decreased secretory material in the thyroid follicular epithelial cells was seen in 8 females at 150 mg/kg/day compared to none in the controls.

In the 1991 Subchronic Toxicity Study in the rat:

- Thyroid hormone levels (T3 and T4) were decreased at the mid (100 mg/kg/day) and high (300 mg/kg/day) dose groups at 6 and 13 weeks. At 6 weeks, T3 levels were decreased (87% not significant) and T4 levels were significantly ($p < 0.05$) decreased (↓30%). At 13 weeks, T3 levels were significantly ($p < 0.05$) decreased (↓66%) and T4 levels were significantly ($p < 0.05$) decreased (↓42%).
- Absolute and relative thyroid weights were significantly ($p < 0.05$) increased (↑168% and ↑126%) at the high dose. Absolute and relative ovarian weights were significantly ($p < 0.05$) increased (58% absolute and 82% relative) at the high dose.

In the Subchronic Toxicity Study [Charles *et al.*, (1996)]:

- T3 concentrations were significantly ($p < 0.05$) decreased in at the mid (100 mg/kg/day) and high (300 mg/kg/day) dose groups with the 2,4-D DMA salt.
- T4 concentrations were significantly ($p < 0.05$) decreased at the mid - and high dose group for the acid, the 2,4-D DMA salt and 2,4-D, 2-EHE.
- Relative thyroid weights were significantly ($p < 0.05$) increased in females at the high dose with the acid, the 2,4-D DMA salt and 2,4-D, 2-EHE.

- The PETA submission provided a brief review of a few human studies that addressed potential reproductive and developmental toxicity of 2,4-D based on the exposure of Vietnam veterans to Agent Orange. The conclusion was that the veterans who had greater potential exposure to Agent Orange did not have an increased risk of fathering babies with major structural birth defects. In fact, even for pesticide applicators and the general population in an agricultural region of Minnesota, a more detailed cross-sectional analysis of this area showed no statistically significant correlation between 2,4-D use and excess adverse birth effects. The Agency

Table 8. Evaluation of Data Submitted in Relation to the Female Pubertal Assay	
Chemical: 2,4-D	**PC Code: 031001**
890.1450 - Female Pubertal Assay (Rat)	
believes that overall, prospective cohort studies still need to be conducted to confirm or test the hypothesis generated by these studies. In addition, additional detail is needed on exposures of the subjects to drugs and chemicals which may have an adverse effect on the male reproductive system that can contribute confounding effects in the outcome and interpretation of these data. While these data provide additional information that upon further investigation may contribute to hazard characterization, they do not provide confirmed or confident linkages between human exposure to 2,4-D and interaction with the estrogen pathway.	
4. Conclusion: The requirement for the **Female Pubertal Assay (890.1450) is satisfied** based on the Extended One-Generation Rat Reproduction Study which provided numerous detailed measures of the endocrine system in developing offspring as a consequence of pre-natal and post-natal exposures to 2,4-D.	

[1] -- = not measured; X = indicates the endpoint was measured in the assay but does not indicate whether or not it satisfies the data requirement of the test order. See section 3 below for a detailed explanation; N/A = not applicable

Table 9. Evaluation of Data Submitted in Relation to the Male Pubertal Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1500 - Male Pubertal Assay (Rat)

1. EDSP Assay Endpoints[1]

Study Type / Literature Citation	MRID No.	Growth	Age and Weight at PPS	Organ Weights[2]										
				TE	EP	SV	VP	DLP	LABC	TH	LI	KDY	ADR	PIT
Evangelista de Duffard *et al.*, (1995)	N/A	--	--	--	--	--	--	--	--	--	--	--	--	--
Evangelista de Duffard *et al.*, (1996)	N/A	--	--	--	--	--	--	--	--	--	--	--	--	--
Florsheim and Velcoff (1962)	48279302	x	--	--	--	--	--	--	--	x	--	--	x	x
Florsheim *et al.*, (1963)	48279303	x	--	--	--	--	--	--	--	--	--	--	--	--
Stoker *et al.*, 2007	N/A	--	--	--	--	--	--	--	--	--	--	--	--	--
Extended 1-Generation Reproduction - Rat	47972101	x	x	x	x	x	--	--	--	x	x	x	x	x
2-Generation Reproduction - Rat	00150557	x	--	x	--	--	--	--	--	--	x	x	--	--
Developmental Toxicity - Acid – 2,4-D Rat	00130407	x	--	--	--	--	--	--	--	--	--	--	--	--
Developmental Toxicity – 2,4-D Salts & Esters– Rat [Charles *et al*, (2001)]	45761204	x	--	--	--	--	--	--	--	--	--	--	--	--
Developmental Toxicity – 2,4-D Acid Rabbit	41747601	x	--	--	--	--	--	--	--	--	--	--	--	--
Developmental Toxicity – 2,4-D Salts& Esters–Rabbit [Charles *et al.*, (2001)]	45761204	x	--	--	--	--	--	--	--	--	--	--	--	--

Table 9. Evaluation of Data Submitted in Relation to the Male Pubertal Assay														
Chemical: 2,4-D							**PC Code: 031001**							
890.1500 - Male Pubertal Assay (Rat)														
Chronic Toxicity/ Carcinogenicity – Rat	43612001	x	--	x	--	--	--	--	--	--	x	x	x	--
Carcinogenicity – Male Mouse	43879801	x	--	x	--	--	--	--	--	--	x	x	--	--
1981: Subchronic Oral toxicity - Rat	00102451	x	--	x	--	--	--	--	--	--	x	x	--	--
1991: Subchronic Oral Toxicity- 2,4-D Acid - Rat	41991501	x	--	x	--	--	--	--	--	x	x	x	--	x
Subchronic Oral Toxicity – 2,4-D Salts & Esters – Rat [Charles *et al.*, (1996)]	45761213	x	--	x	x	--	--	--	--	x	x	x	x	x
Subchronic Oral Toxicity - Mouse	41991502	x	--	x	--	--	--	--	--	x	x	x	x	x
Subchronic Inhalation Toxicity- Rat	47398701	x	--	x	x	--	--	--	--	--	--	--	x	--
Epidemiology Studies (PETA OSRI)	N/A	--	--	--	--	--	--	--	--	--	--	--	--	--
Study Type/ Literature Citation	**MRID No.**	**EDSP Assay Endpoints: Clinical Chemistry and Pathology[1]**												
		Blood Chemistry	**Hormones**			**Histopathology**								
			Testosterone	**T4**	**TSH**	**Epididymides**	**Testes**	**Thyroid**	**Kidney**					
Evangelista de Duffard *et al.*, (1995)	N/A	--	--	--	--	--	--	--	--					
Evangelista de Duffard *et al.*, (1996)	N/A	--	--	--	--	--	--	--	--					
Florsheim and Velcoff (1962)	48279302	--	--	--	--	--	--	--	--					

Table 9. Evaluation of Data Submitted in Relation to the Male Pubertal Assay									
Chemical: 2,4-D					**PC Code: 031001**				
890.1500 - Male Pubertal Assay (Rat)									
Florsheim *et al.*, (1963)	48279303	--	--	--	--	--	--	--	--
Stoker *et al.*, 2007	N/A	--	--	--	--	--	--	--	--
Extended 1-Generation Reproduction - Rat	47972101	x	x	x	x	x	x	x	x
2-Generation Reproduction – Rat	00150557	x	--	--	--	x	x	x	x
Developmental Toxicity - Acid - 2,4-D Rat	00130407	--	--	--	--	--	--	--	--
Developmental Toxicity – 2,4-D Salts & Esters – Rat [Charles *et al.*, (2001)]	45761204	--	--	--	--	--	--	--	--
Developmental Toxicity – 2,4-D Acid Rabbit	41747601	--	--	--	--	--	--	--	--
Developmental Toxicity – 2,4-D Salts& Esters – Rabbit [Charles *et al.*, (2001)]	45761204	--	--	--	--	--	--	--	--
Chronic toxicity/ Carcinogenicity – Rat	43612001	x	--	x	--	x	x	x	x
Carcinogenicity – Male Mouse	43879801	--	--	--	--	x	x	x	x
1981: Subchronic Oral toxicity - Rat	00102451	x	--	x	--	x	x	x	x
1991: Subchronic Oral Toxicity- 2,4-D Acid - Rat	41991501	x	--	x	--	x	x	x	x
Subchronic Oral Toxicity – 2,4-D Salts & Esters – Rat [Charles *et al.*, (1996)]	45761213	--	--	--	--	x	x	x	x

Table 9. Evaluation of Data Submitted in Relation to the Male Pubertal Assay									
Chemical: 2,4-D					**PC Code: 031001**				
890.1500 - Male Pubertal Assay (Rat)									
Subchronic Oral Toxicity - Mouse	41991502	--	--	x	--	x	x	x	x
Subchronic Inhalation Toxicity- Rat	47398701	--	--	--	--	x	x	x	x
Epidemiology Studies (PETA OSRI)	N/A	--	--	--	--	--	--	--	--

2. Summary of Study Findings:

Study Type / Literature Citation	MRID No.	Findings
Evangelista Duffard *et al.*, (1995)	N/A	See Section IV of this report.
Evangelista Duffard *et al.*, (1996)	N/A	See Section IV of this report.
Florsheim and Velcoff (1962)	48279302	Male Sprague-Dawley rats on a restricted iodine diet regimen for 26 days with a daily subcutaneous injection of 1 cc iodine solution (0.8 µc Iodine 131 and 5 or 10 µg stable iodide). Experimental rats were subcutaneously injected with 80 mg/kg/day 2,4-D for the last 7 days of the regimen. After autopsy, a 3 cc serum sample from each animal was evaluated. A significant increase in 24-hour iodine-131 (I131) uptake and significant decreases in serum protein-bound-iodine (PBI) and thyroid:serum radioiodide ratio were observed. However, 2,4-D exposure had no effect on serum or pituitary TSH concentrations, thyroidal cell height, or thyroid histopathology. Therefore, these findings provide a possible mechanistic explanation for decreases in circulating thyroid concentrations in rats at high dosages of 2,4-D, but do not provide evidence of a biologically significant adverse effect.
Florsheim *et al.*, (1963)	48279303	This study evaluated the distribution of thyroxine after 2,4-D exposure. Male Sprague-Dawley rats on a restricted iodine diet regimen with fixed dose iodine replenishment, with subcutaneously injected 80 mg/kg/day 2,4-D for the last 7 days of the regimen A significant increase in 24-hour I131 uptake and significant decreases in serum protein bound iodine (PBI) and thyroid: serum radioiodide ratios were observed. A 50% decrease in protein-bound iodine was reported with subcutaneous administration of 80 mg/kg/day 2,4-D to male rats for 7 days. However, 2,4-D exposure had no effect on serum or

Table 9. Evaluation of Data Submitted in Relation to the Male Pubertal Assay		
Chemical: 2,4-D		**PC Code: 031001**
890.1500 - Male Pubertal Assay (Rat)		
		pituitary TSH concentrations, thyroid follicular cell height, or thyroid histopathology. The authors concluded that "2,4-D reduces the serum binding capacity for thyroxine" and "2,4-D lowers serum thyroxine binding by competing with thyroxine for its binding sites, although it appears to be a weak competitor."
Stoker *et al.*, 2007	N/A	See Section IV of this report.
Extended 1-Generation Reproduction - Rat	47972101	In the P1 animals, male mating, fertility, and gestation indices were comparable among the groups, and post-implantation loss was comparable among the groups. Both the time to mating and gestation length was comparable among the groups. The decreases observed in the weights of the testes, epididymides, seminal vesicles and the prostate at the mid and high dose groups were not attributed to treatment since the values in the concurrent control were outside of the laboratory historical control range. There were no treatment-related effects on sperm motility or progressive motility, no differences in testicular spermatid and epididymal sperm counts, and no differences in the proportion of abnormal sperm. In the F1 Offspring (PND 70), there were no treatment-related effects on the numbers of live or dead F1 pups born/litter or on pup survival or sex ratio. There was no significant, treatment-related difference in absolute or relative anogenital distance in male pups and no differences in nipple/areolae retention between control and high-dose groups in male pups. Male pups at the high dose displayed a slight delay in preputial separation (1.6 days), which was accompanied by a very slight reduction in body weight compared to the control (↓2.1 grams; 99% of control). Although statistical significance was not attained, treatment-related changes observed in the absolute weights of the prostate (↓6%), epididymides (↓6%) and the thyroid (↓11%), adrenal (↓12%) and the pituitary (↓14%) glands in males at the high dose. No histopathological lesions were seen in the testes, epididymides, seminal vesicle and the prostate glands. In the pups culled on PND 4, there were no treatment-related effects in serum T3, T4 and TSH levels. In the PND 22 pups, decreases in T3 (mid and high dose males) and T4 (high dose males) and an increase in TSH (mid dose males) levels were seen. Thyroid weight and histopathology data were not collected for this age group. In the PND 62-64 offspring, TSH level was increased in the mid and high dose males but not in a dose-dependent manner. Decreased T4 was observed only in males at the high

Table 9. Evaluation of Data Submitted in Relation to the Male Pubertal Assay		
Chemical: 2,4-D		**PC Code: 031001**
890.1500 - Male Pubertal Assay (Rat)		
		dose, and decreased T3 was observed at all dose levels in males but there was no dose-response. Absolute thyroid weights were decreased (↓11) at the high dose, but terminal body weight was also decreased (↓10). There were no histopathological findings reported in the thyroids. In the F1 Offspring (PND139), no treatment-related effects were seen on sperm motility or progressive motility, no differences in testicular spermatid and epididymal sperm counts, and no differences in the proportion of abnormal sperms. No treatment-related histopathological lesions were seen in the testes, epididymides, seminal vesicles, prostate, ovaries, oviducts, uterus, vagina, cervix, mammary, thyroid, adrenal and pituitary glands of PND 139 animals. No histopathological lesions were seen in the testes, epididymides, seminal vesicles, prostate, thyroid, adrenal or pituitary glands.
2-Generation Reproduction - Rat	00150557	In the F0 generation, no apparent adverse effect was observed on fertility. Pre-coital intervals were comparable among the groups. The duration of gestation was increased (0.6 days) at the high-dose of F0 rats producing the F1b pups. The gestation survival index was comparable among the groups for the F1a pups but was significantly decreased for the F1b litters. There was a significant decrease in the number of F1a female fetuses at the high-dose. The number of F1b pups born dead/dying by day 1 was significantly increased at the high-dose. Fla litter size was slightly lower at the high-dose, but F1b litter size was significantly lower than the control. Fla pup viability was comparable throughout weaning, but the Flb pup viability was significantly lower throughout the weaning period. There was a significant decrease in Flb pup survival to lactation day 4 at the high-dose level as well as survival to lactation day 28. In the F1 generation, no apparent adverse effect was observed on fertility at either dose level. Pre-coital intervals and gestation lengths were comparable among the groups. The gestation survival index and the viability index were comparable among the groups for both the F2a and F2b litters. Litter size, body weights, and the sex ratio were comparable among the groups in both the F2a and F2b litters. No treatment-related changes in absolute or relative testes weights nor were there any treatment-related histopathological lesions seen in the testes of the offspring of any generation.
Developmental Toxicity - 2,4-D Acid - Rat	00130407	No treatment-related changes were seen in pregnancy rate, number of corpora lutea, mean number of implantations/litter, post-implantation loss, early or late resorptions, number of live fetuses/litter, fetal sex ratio, or soft tissue abnormalities at any dose.

Table 9. Evaluation of Data Submitted in Relation to the Male Pubertal Assay		
Chemical: 2,4-D		**PC Code: 031001**
890.1500 - Male Pubertal Assay (Rat)		
Developmental Toxicity – 2,4-D Salts & Esters – Rat [Charles *et al.*, (2001)]	45761204	No treatment-related changes were seen in pregnancy rate, number of corpora lutea, mean number of implantations/litter, post-implantation loss, early or late resorptions, number of live fetuses/litter, fetal sex ratio, or soft tissue abnormalities for 2,4-D acid or any of its amine salts (2,4-D DMA; 2.4-D DEA; 2,4-D IPA; 2.4-D TIPA) or esters (2.4-D BEE, 2.4-D EHE and 2,4-DIPE).
Developmental Toxicity – 2,4-D Acid Rabbit	41747601	No treatment-related changes were seen in pregnancy rate, number of corpora lutea, mean number of implantations/litter, post-implantation loss, early or late resorptions, number of live fetuses/litter, fetal sex ratio, or soft tissue abnormalities at any dose.
Developmental Toxicity – 2,4-D Salts& Esters – Rabbit [Charles *et al.*, (2001)]	45761204	No treatment-related changes were seen in pregnancy rate, number of corpora lutea, mean number of implantations/litter, post-implantation loss, early or late resorptions, number of live fetuses/litter, fetal sex ratio, or soft tissue abnormalities for 2,4-D acid or any of its amine salts (2,4-D DMA; 2.4-D DEA; 2.4-D IPA; 2.4-D TIPA) or esters (2.4-D BEE and 2.4-D EHE).
Chronic Toxicity/ Carcinogenicity – Rat	43612001	No treatment-related changes were seen in T3 concentrations at any dose. Thyroxin (T4) levels were decreased at all intervals measured (6, 12, 18 and 24 months) with the decreases reaching statistical significance ($p < 0.05$) only at 12 and 24 months. At 12 months, the decreases were -14% and -70% at the 75 and 150 mg/kg/day, respectively. At 24 months, the decreases were -32% and -64% in males at 75 and 150 mg/kg/day, respectively. No treatment-related changes were seen in absolute or relative adrenal weights. Treatment-related changes were seen in the testes: absolute weights were significantly ($p<0.05$) decreased (15%) at 150 mg/kg/day at the interim (12-month) sacrifice and both the absolute (52%) and relative (49%) weights were decreased at 150 mg/kg/day at the terminal (24-months) sacrifices. Absolute and relative thyroid weights were significantly ($p < 0.05$) increased at 12 and 24 months. No treatment-related histopathological lesions were seen in the epididymides, seminal vesicles, prostate, thyroid, adrenal and pituitary glands. Atrophy of the testes was seen in 2/50 at 150 mg/kg/day compared to 0/50 in the controls.
Carcinogenicity – Male Mouse	43879801	Male reproductive organ weights were not evaluated. No treatment-related histopathological lesions were seen in the testes, epididymides, seminal vesicles, prostate, thyroid, adrenal or pituitary glands.
1981: Subchronic Oral toxicity - Rat	00102451	No treatment-related changes in thyroid hormone levels, absolute or relative testes weight were seen. No treatment-related histopathological lesions were seen in the testes, epididymides, accessory sex glands, prostate, thyroid, adrenal or pituitary glands.

Table 9. Evaluation of Data Submitted in Relation to the Male Pubertal Assay		
Chemical: 2,4-D		**PC Code: 031001**
890.1500 - Male Pubertal Assay (Rat)		
1991: Subchronic Oral Toxicity-2,4-D Acid - Rat	41991501	Thyroid hormones (decreased T3 and T4 levels) were observed at 100 and 300 mg/kg/day at 6 and 13 weeks. Significant ($p < 0.05$) decreases in T3 (63% of control; $p<0.05$) and T4 (24%) levels were seen at 6 weeks and also at week 13 [(T3 (73%) and T4 (26%)]. Absolute and relative testes weights were significantly ($p < 0.05$) increased (51% absolute and 67% relative) at the high dose. Absolute and relative thyroid weights were significantly ($p < 0.05$) increased in males (140% absolute and 186% relative) at the high dose. Relative adrenal weight was significantly ($p < 0.05$) increased (130% of control) in males at the high dose. Treatment-related histopathological changes were atrophy of the testes (8/10) and hypertrophy of the zona glomerulosa of the adrenal cortex in (8/10) at the high dose.
Subchronic Oral Toxicity – 2,4-D Salts & Esters – Rat [Charles *et al.*, (1996)]	45761213	T3 concentrations were significantly ($p < 0.05$) decreased in males at the high dose with the acid and the 2,4-D DMA salt. T4 concentrations were significantly ($p < 0.05$) decreased at the mid and high dose groups for the acid and at the high dose with the 2,4-D DMA salt and 2,4-D 2-EHE. Relative thyroid weights were significantly ($p < 0.05$) increased at the mid and high dose groups with the acid; at the high dose with the 2,4-D DMA salt and 2,4-D 2- EHE. Relative testes weights were significantly ($p < 0.05$) decreased in the high dose for all three compounds. No treatment-related changes in absolute or relative weights of the adrenals or pituitary glands were seen. Histopathology revealed hypertrophy of the adrenal cortex at the high dose with the acid (8/10), 2,4-D DMA salt (10/10), and 2,4-D 2-EHE (6/10). No treatment-related histopathological lesions were seen in the testes, epididymides thyroid and pituitary glands.
Subchronic Oral Toxicity - Mouse	41991502	No treatment-related changes in absolute or relative weights of the testes, thyroid, adrenals or pituitary glands. No treatment-related histopathological lesions were seen in the testes, epididymides, thyroid, adrenal or pituitary glands.
Subchronic Inhalation Toxicity- Rat	47398701	No treatment-related changes in absolute or relative weights of the testes and adrenals glands. No treatment-related histopathological lesions were seen in the testes, epididymides, seminal vesicles, prostate, thyroid, adrenal or pituitary glands.
Epidemiology Studies (PETA OSRI)	N/A	See discussion below in Section 3.

Table 9. Evaluation of Data Submitted in Relation to the Male Pubertal Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1500 - Male Pubertal Assay (Rat)

3. Agency's Evaluation of the OSRI:

The submission provided by the Test Order Recipient stated that "The male pubertal assay is intended to characterize androgenic or anti-androgenic effects of the test compound. Estrogenic effects may also be observed in this assay. In addition, it evaluates parameters relevant to thyroid, adrenal, renal, hepatic and pituitary structure or function. *In vitro* ToxCast® studies conducted under the auspices of the US EPA and cited as OSRI showed no evidence of 2,4-D interaction with the androgen, estrogen or thyroid receptors. Review of published in vitro data across studies confirms the lack of androgen or estrogen receptor binding or transactivation in several test systems. As discussed in the text and summarized in the matrix for the Male Pubertal assay in Appendix II, review of the reproductive toxicity and subchronic and chronic toxicity regulatory toxicity data for 2,4-D cited as OSRI, as well as other supplementary studies, do not show any consistent patterns of effects related to estrogenicity or androgenicity at any dose, or to anti-androgenicity at doses not exceeding the threshold for saturation of renal clearance. 2,4-D was evaluated in two reproductive toxicity studies including an F1-extended one generation study that incorporated multiple endocrine endpoints. These evaluations overlapped the exposure time frame for the male pubertal assay, and arguably the reproductive study design (conventional and F1-extended) tests a more sensitive timeframe, due to the perinatal exposure in this study paradigm. "Parameters evaluated in theF1-extended one generation study included many very sensitive to anti-androgenicity or androgenicity, including balano-preputial separation, anogenital distance, quantitative nipple retention, evaluation of sperm parameters, reproductive organ weights and detailed histopathological evaluations of male reproductive organs. There were no effects of 2,4-Don androgen or estrogen sensitive parameters in this study (Marty *et al.,* 2010). One study(available as abstract only) in the published literature (Stoker *et al.,* 2007) reported an anti androgenic pattern of effects in a male pubertal assay at a very high dose far exceeding the KMD; however, no anti-androgenic effects were seen at the lower dose, which still exceeded the KMD. A number of parameters related to thyroid function were measured in the 2,4-D studies conducted for pesticide registration purposes. The F1-extended one generation study evaluated thyroid hormones (T3, T4 and TSH), weight and histopathology across life stages, and also evaluated auditory startle and brain morphometry data in the DNT component of the study. There were no exposure related effects on the thyroid of males in this study (Marty *et al.,* 2010). Exposure related effects in male rodents in other studies were limited to decreases in T4 at high doses exceeding the KMD; no histopathological findings correlated with the hormonal change (Schulze [1991a]; Gorzinski *et al.* [1981a]; Jeffries *et al.,* [1995]). No robust effects on auditory startle or statistically significant brain morphometric changes were seen in the DNT component of the extended one-generation reproductive toxicity study (Marty *et al.,* 2010), which would be expected sequelae to any marked thyroid hormone deficiency. Myelin deposition was also characterized at PND 22and 60, and no exposure-related changes were observed. Overall, there are no findings suggesting an effect on the thyroid at doses below the saturation threshold for renal clearance. Duffard's group in Argentina has reported a number of studies of developmental or adult neurotoxicity of 2,4-D; these studies are high dose evaluations and many suffer from methodological deficiencies which make interpretation and reliability questionable. The findings in these studies were not replicated in the F1-extended one generation study, which

Table 9. Evaluation of Data Submitted in Relation to the Male Pubertal Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1500 - Male Pubertal Assay (Rat)

had much more well-characterized exposures, and, as noted, evaluated a number of DNT parameters. NOAELs have been established for the rodent thyroid effects."

"Mechanistic studies indicated displacement of thyroid hormone from thyroid plasma binding proteins as the primary mode of action for high-dose specific changes in circulating thyroid hormones. It should be noted that the potential for thyroid effects will be further evaluated in the amphibian metamorphosis assay (AMA), which the 2,4-D Task Force is committing to conduct. This AMA also provides the redundancy appropriate to a screening battery. Adrenal histopathology showed hypertrophy in the zona glomerulosa of the adrenal cortex at a very high dose in a subchronic rat study (Schulze, 1991a); no adrenal effects were seen in the chronic rat study (Jeffries *et al.,* 1995). The zona glomerulosa is not associated with sex hormone synthesis. Pituitary weight changes were seen at high doses in several rodent studies; the direction of the change was inconsistent and there was no correlating histopathology suggesting an exposure related finding. Renal and hepatic toxicity have been thoroughly characterized in the extant data base. In *toto,* no effects were seen in males that were likely to be related to estrogen or androgen hormone modulation at doses not exceeding a KMD. Thyroid findings in males were limited to decreased T4 at very high doses exceeding a KMD, and lacking any correlating histopathological findings. No consistent pattern of effects was seen in evaluations of the adrenal or pituitary. The potential for thyroid effects will be further evaluated in the amphibian metamorphosis assay, which the 2,4-D Task Force is committing to conduct. In view of the extensive evaluations of the potential influence of 2,4-D on the male hormonal system and reproductive toxicity, and the lack of reliable or consistent indicators of any adverse endocrine-mediated effects at doses not exceeding the saturation threshold for renal clearance, we consider the male pubertal assay redundant and wasteful of both animal lives and testing resources. Based on the extensive data available and submitted as OSRI, the 2,4-D Task Force requests a waiver from a Male Pubertal assay."

The PETA comments submitted to the Agency in response to the EDSP screening order issued for 2,4-D stated that "In summary, 2,4-D is an extremely well-studied chemical. Its endocrine disrupting potential has already been addressed in mammalian subchronic, reproductive and developmental studies as well as in numerous in vitro assays, in chronic studies in birds, fish and reptiles and in epidemiologic studies. Further, a study designed in consultation with the EPA to definitively address remaining endocrine-sensitive endpoints is nearing completion. There is no need for further testing under the EDSP."

Table 9. Evaluation of Data Submitted in Relation to the Male Pubertal Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1500 - Male Pubertal Assay (Rat)

On review of the OSRI submitted, the Agency noted the following observations with regard to the androgen pathway:

In the Extended One-Generation Reproduction Study:

- There were no significant effects on any of the reproductive indices in the P1 rats at any dose level. Male mating, conception, fertility, and gestation indices, the percent post-implantation loss, and the sex ratio were comparable among the groups.
- There were no significant treatment related effects on sperm counts, sperm morphology or sperm motility in P1 and F1 offspring.
- There were no significant treatment-related differences in anogenital distance (AGD) in either sex in the F1 generation.
- There was a slight delay (1.6 days) in preputial separation at the high dose (40 mg/kg/day) with a slight (2.1 g; 99% of control) in body weight.
- External male genitalia were normal at birth and there was no difference in nipple/areolae retention in male pups.
- No treatment-related effects were seen in the absolute or relative weights of the androgen sensitive organs (testes, epididymides, seminal vesicles and prostate) in P1 or F1 offspring.
- No treatment-related histopathological lesions were seen in any of the androgen sensitive organs (testes, epididymides, seminal vesicles and prostate) of P1 or F1 offspring.
- No treatment-related effects were seen in the absolute or relative weights or histopathology of the pituitary glands in P1 or F1 offspring.

In the Chronic Toxicity/Carcinogenicity Study in the rat:

- Testes weights were significantly decreased in rats at the high dose at both the interim (absolute weights) and terminal (absolute and relative weights) sacrifices, and
- Atrophy of the testes was seen in 2/50 at 150 mg/kg/day compared to 0/50 in the controls.

In the Subchronic Toxicity Study in the rat (MRID 41991501) :

- Absolute and relative testes weights were significantly increased at the high dose, and
- Atrophy of the testes was seen in 8/10 at the high dose compared to 0/10 in the controls.

Table 9. Evaluation of Data Submitted in Relation to the Male Pubertal Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1500 - Male Pubertal Assay (Rat)

On review of the OSRI submitted, the Agency noted the following observations with regard to the thyroid pathway:

In the Extended One-Generation Reproduction Study:

- In the F1 PND 4 pups, there were no statistically-significant differences in serum T3, T4, or TSH in PND 4 culled pups. T4 was reduced (not statistically significant) to a similar extent at the mid (↓14%) and high (↓12%) dose levels. Thyroid weight and histopathology data were not collected for this age group.
- In the F1 PND 22 weanlings, males exhibited decreases in T3 levels at the mid-(↓19%) and high (↓13%) dose groups, but there was no dose response. T4 levels were decreased at the mid (↓18%) dose and a statistically-significant reduction (↓28%) at the high dose. Thyroid weight and histopathology data were not collected for this age group.
- In the F1 PND 62-64, T3 levels were decreased at the mid (↓15%) and high (↓8%) dose groups. T4 was decreased (↓12-13%) at the mid and high dose groups. TSH was increased at the mid (↑26%) and high (↑23%) dose groups.
- PND 70 offspring displayed decreased absolute thyroid weights (↓11) at 40 mg/kg/day, but terminal body weight was also decreased (↓10).

In the Chronic Toxicity/Carcinogenicity Study in the rat:

- No treatment-related changes were seen in T3 concentrations at any dose.
- Thyroxin (T4) levels were decreased at all intervals measured (6, 12, 18 and 24 months) with the decreases reaching statistical significance ($p < 0.05$) only at 12 and 24 months. At 12 months, the decreases were -14% and -70% at the 75 and 150 mg/kg/day, respectively. At 24 months, the decreases were -32% and -64% in males at 75 and 150 mg/kg/day, respectively.
- Absolute and relative thyroid weights were significantly ($p < 0.05$) increased at 12 and 24 months.
- No treatment-related histopathological lesions were seen in the thyroid glands.

In the 1991 Subchronic Toxicity Study in the rat (MRID 41991501):

- Thyroid hormones (decreased T3 and T4 levels) were observed at the mid and high dose levels at 6 and 13 weeks. Significant ($p < 0.05$) decreases in T3 (63% of control; $p<0.05$) and T4 (24%) levels were seen at 6 weeks and also at week 13 [(T3 (73%) and T4 (26%)].
- Absolute and relative thyroid weights were significantly ($p < 0.05$) increased in males (140% absolute and 186% relative) at the high dose.
- No treatment-related histopathological lesions were seen in the thyroid glands.

Table 9. Evaluation of Data Submitted in Relation to the Male Pubertal Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1500 - Male Pubertal Assay (Rat)

In the Subchronic Toxicity Study with 2,4-D [Charles *et al.*, (1996)]:

- T3 concentrations were significantly ($p < 0.05$) decreased in males at the high dose with the acid and the 2,4-D DMA salt. T4 concentrations were significantly ($p < 0.05$) decreased at the mid and high dose groups for the acid and at the high dose with the 2,4-D DMA salt and 2,4-D 2-EHE.
- Relative thyroid weights were significantly ($p < 0.05$) increased at the mid and high dose groups with the acid and at the high dose with the 2,4-D DMA salt and 2,4-D 2- EHE.
- No treatment-related histopathological lesions were seen in the thyroid glands.

- The PETA submission provided a brief review of a few human studies that addressed potential reproductive and developmental toxicity of 2,4-D based on the exposure of Vietnam veterans to Agent Orange. The conclusion was that the veterans who had greater potential exposure to Agent Orange did not have an increased risk of fathering babies with major structural birth defects. The submission also stated that, even for pesticide applicators and the general population in an agricultural region of Minnesota, a more detailed cross-sectional analysis of this area showed no statistically significant correlation between 2,4-D use and excess adverse birth effects. The Agency believes that overall, prospective cohort studies still need to be conducted to confirm or test the hypothesis generated by these studies. In addition, additional detail is needed on exposures of the subjects to drugs and chemicals which may have an adverse effect on the male reproductive system that can contribute confounding effects in the outcome and interpretation of these data. While these data provide additional information that upon further investigation may contribute to hazard characterization, they do not provide confirmed or confident linkages between human exposure to 2,4-D and interaction with the androgen pathway.

4. Conclusion: The requirement for the **Male Pubertal Assay (890.1500) is satisfied** based on the Extended One-Generation Rat Reproduction Study which provided numerous detailed measures of the endocrine system in developing offspring as a consequence of pre-natal and post-natal exposures to 2,4-D.

[1] -- = not measured; X = indicates the endpoint was measured in the assay but does not indicate whether or not it satisfies the data requirement of the test order. See section 3 below for a detailed explanation; N/A = not applicable

[2] TE = Testes, EP = Epididymides; SV = Seminal Vesicle; VP= Ventral Prostate; DLP= Dorsolateral Prostate; LABC= levator ani-bulbocavernosus muscle complex; TH= Thyroid; LI= Liver; KDY = Kidney; ADR= Adrenal; PIT= Pituitary

Table 10. Evaluation of Data Submitted in Relation to the Steroidogenesis Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1550 - Steroidogenesis Assay (Human Cell Line – H295R)

1. EDSP Assay Endpoints[1]

Study Type / Literature Citation	MRID No.	17β- Estradiol Content	Testosterone Content	Cell Viability
Orton *et al.*, (2009)	48041727	x	x	--
Extended 1-Generation Reproduction - Rat	47972101	--	--	--
2-Generation Reproduction - Rat	00150557	--	--	--
Developmental Toxicity- Acid – 2,4-D Rat	00130407	--	--	--
Developmental Toxicity- 2,4-D Salt & Esters – Rat [Charles *et al.*, (2001)]	45761204	--	--	--
Developmental Toxicity- 2,4-D Acid -Rabbit	41747601	--	--	--
Developmental Toxicity –2,4-D Salt & Esters – Rabbit [Charles *et al.*, (2001)]	45761204	--	--	--
Chronic Toxicity /Carcinogenicity – Rat	43612001	--	--	--
Carcinogenicity – Female Mouse	43879801	--	--	--
1981: Subchronic Oral toxicity – Rat	00102451	--	--	--
1991: Subchronic Oral Toxicity- Acid - 2,4-D Rat	41991501	--	--	--
Subchronic Oral Toxicity – 2,4-D Salts & Esters – Rat [Charles *et al.*, (1996)]	45761213	--	--	--
Subchronic Oral Toxicity -Mouse	41991502	--	--	--
Subchronic Inhalation Toxicity- Rat	47398701	--	--	--
Avian Reproduction Study with Bobwhite Quail (*Colinus virginianus*)	45336401	--	--	--
Epidemiology Studies (PETA OSRI)	N/A	--	--	--

Table 10. Evaluation of Data Submitted in Relation to the Steroidogenesis Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1550 - Steroidogenesis Assay (Human Cell Line – H295R)

2. Summary of Study Findings:

Study Type / Literature Citation	MRID No.	Findings
Orton *et al.*, (2009)	48041727	See discussion below in Section 3.
Epidemiology Studies (PETA OSRI)	N/A	See discussion below in Section 3.
Part 158 studies cited above	See above	The cited *in vivo* Part 158 mammalian toxicity studies do measure the ability of the chemical to interfere with steroid hormone synthesis.

3. Agency's Evaluation of the OSRI:

The submission provided by the Test Order Recipient stated that "This assay evaluates the potential of a compound to inhibit steroidogenesis. Orton *et al.* (2009) performed an amphibian steroidogenesis assay with 2,4-D and found no effects on testosterone production. As noted above, several investigators have found no 2,4-D effects on aromatase inhibition, which is the second major step in steroidogenesis to produce estrogen, in alligators (studies discussed in Appendix III). Further, the extensive mammalian toxicity data base shows no downstream effects characteristic of impaired steroidogenesis at doses less than the KMD or threshold for renal clearance saturation. The 2,4-D Task Force therefore requests a waiver for the Steroidogenesis assay."

The PETA comments submitted to the Agency in response to the EDSP screening order issued for 2,4-D stated that "In summary, 2,4-D is an extremely well-studied chemical. Its endocrine disrupting potential has already been addressed in mammalian subchronic, reproductive and developmental studies as well as in numerous *in vitro* assays, in chronic studies in birds, fish and reptiles and in epidemiologic studies. Further, a study designed in consultation with the EPA to definitively address remaining endocrine-sensitive endpoints is nearing completion. There is no need for further testing under the EDSP."

On review of the OSRI submitted, the Agency noted the following:

- Because *in vivo* mammalian toxicity studies may not confirm that downstream effects can be clearly linked to impaired steroid hormone synthesis and yet existing data do not address whether exposure of non-mammalian species to 2,4-D would result in impaired steroidogenesis. EPA has required an *in vitro* study on the effects of 2.4 -D on steroid hormone synthesis. Estrogen is at the end of the steroid hormone synthesis pathway and intermediate hormone levels, such as testosterone levels, may be affected without noted effects on estrogen levels.

Table 10. Evaluation of Data Submitted in Relation to the Steroidogenesis Assay	
Chemical: 2,4-D	**PC Code: 031001**

890.1550 - Steroidogenesis Assay (Human Cell Line – H295R)

- Orton *et al* (2009) measured steroid hormone synthesis (testosterone and estrogen) in ovary tissue slices of *X. laevis* at two concentrations of test substance. 2,4-D was reported negative, but no data were provided. This assay is not a substitute for 890.1550 which uses multiple concentrations in a cell line that possesses all of the enzymes required for steroid hormone synthesis. The authors used an unusual protocol that measures ovulation as an endpoint, rather than hormones. It provided no measurement of testosterone production. In contrast, the H295R assay measures the amount of testosterone and estrogen produced. The assay has not been validated or widely used, and it resembles the sliced testes assay which EPA rejected because variability was too high.

- The PETA submission provided a brief review of a few human studies that addressed potential reproductive and developmental toxicity of 2,4-D based on the exposure of Vietnam veterans to Agent Orange. The conclusion was that the veterans who had greater potential exposure to Agent Orange did not have an increased risk of fathering babies with major structural birth defects. The submission also noted that, even for pesticide applicators and the general population in an agricultural region of Minnesota, a more detailed cross-sectional analysis of this area showed no statistically significant correlation between 2,4-D use and excess adverse birth effects. The Agency believes that overall, prospective cohort studies still need to be conducted to confirm or test the hypothesis generated by these studies. In addition, additional detail is needed on exposures of the subjects to drugs and chemicals which may have an adverse effect on the male reproductive system that can contribute confounding effects in the outcome and interpretation of these data. While these data provide additional information that upon further investigation may contribute to hazard characterization, they do not provide confirmed or confident linkages between human exposure to 2,4-D and interaction with the endocrine system.

4. Conclusion:

Based on the deficiencies discussed above, the data cited as OSRI did not satisfy the requirement for the Steroidogenesis Assay using Guideline 890.1550.

[1] -- = not measured; X = indicates the endpoint was measured in the assay but does not indicate whether or not it satisfies the data requirement of the test order. See section 3 below for a detailed explanation; N/A = not applicable

Table 11. Evaluation of Data Submitted in Relation to the Uterotrophic Assay		
Chemical: 2,4-D		**PC Code: 031001**
890.1600 - Uterotrophic Assay (Rat)		
1. EDSP Assay Endpoints[1]		
Study Type / Literature Citation	**MRID No.**	**Uterus Weight**
Evangelista deDuffard *et al*, (1990)	N/A	--
Petit *et al,* (1997)	N/A	--
Xie *et al.*, (2005)	N/A	--
Extended 1-Generation Reproduction - Rat	47972101	x
2-Generation Reproduction - Rat	00150557	--
Developmental Toxicity- 2,4-D Acid -Rat	00130407	--
Developmental Toxicity- 2,4-D Salt & Esters – Rat [Charles *et al.*, (2001)]	45761204	--
Developmental Toxicity- 2,4-D Acid -Rabbit	41747601	--
Developmental Toxicity –2,4-D Salt & Esters - Rabbit [Charles *et al.*, (2001)]	45761204	--
Chronic Toxicity /Carcinogenicity – Rat	43612001	--
Carcinogenicity – Female Mouse	43879801	--
1981: Subchronic Oral toxicity - 2,4-D Rat	00102451	--
1991: Subchronic Oral Toxicity- 2,4-D Acid - Rat	41991501	--
Subchronic Oral Toxicity – 2,4-D Salts & Esters – Rat [Charles *et al.*, (1996)]	45761213	--
Subchronic Oral Toxicity -Mouse	41991502	--
Subchronic Inhalation Toxicity- Rat	47398701	--
Avian Reproduction Study with Bobwhite Quail (*Colinus virginianus*)	45336401	--
Epidemiology studies (PETA)	N/A	--

Table 11. Evaluation of Data Submitted in Relation to the Uterotrophic Assay		
Chemical: 2,4-D		**PC Code: 031001**
890.1600 - Uterotrophic Assay (Rat)		
2. Summary of Study Findings:		
Study Type / Literature Citation	**MRID No.**	**Findings**
Evangelista de Duffard *et al.*, (1990)	N/A	See Section IV of this report.
Petit *et al.* (1997)	N/A	Petit *et al.*, (1997) tested 2,4-D in three different in vitro assays. It was tested in yeast a transactivation assay over a range of 10^{-8} to 10^{-4} M in which the yeast was transfected with rainbow trout ERα using a β-galactosidase reporter. Similarly a competitive binding assay was run using yeast cells and rainbow trout ERα. It was also tested for its ability to induce vitellogenin mRNA in a primary rainbow trout hepatocyte culture. The assay results were reported as follows: β-Gal expression in the TA assay = 17.95%, 8.0% Vg induction, and fold induction needed to displace 50% of bound E2 > 10,000. All results were described as negative.
Xie *et al,* (2005)	N/A	This study evaluated vitellogenin induction in immature trout exposed to 2,4-D. Although positive findings were reported, this study had significant endpoint response variability and the findings were inconsistent with *in vitro* studies suggesting a lack of ER binding or activation in trout as reported by Petit *et al* 1997 as discussed above.
Extended 1-Generation Reproduction - Rat	47972101	In the P1 animals, female mating, conception, fertility, and gestation indices were comparable among the groups, and post-implantation loss was comparable among the groups. Both time to mating and gestation length were comparable among the groups. There were no alterations in estrous cycle pattern in females at the high dose, and no significant difference in mean estrous cycle length in P1 females at any dose level compared to the control. There was a non-statistically significant increase in uterine weights (↑17%, both absolute and relative) at the high dose.

Table 11. Evaluation of Data Submitted in Relation to the Uterotrophic Assay		
Chemical: 2,4-D		**PC Code: 031001**
890.1600 - Uterotrophic Assay (Rat)		
		In the GD 17 females, reproductive indices and the numbers of corpora lutea and implantations were comparable among the groups. There was a slight increase in resorptions at the high dose (0.9 vs 1.5) although there was wide variability (standard deviations exceed the means). There was a slight increase in post-implantation loss at the high dose (9.2 vs 5.5). In the F1 offspring (PND 70), there was no significant, treatment-related difference in absolute or relative anogenital distance in either sex and no differences in nipple/areolae retention between control and high-dose groups in either sex. The age at vaginal opening was comparable among the groups of F1 females. In the F1 offspring (PND139), no treatment-related differences were observed in mean estrous cycle length at any dose level. There were no significant, treatment-related effects on the numbers of small follicles, growing follicles, or total follicles. Uterine weights were increased at the mid (↑10% absolute and ↑10% relative) and high (↑10% absolute and ↑11% relative) dose groups. Ovarian follicle counts were comparable between the control and high dose females. No treatment-related histopathological lesions were seen in the ovaries, oviducts, uterus, vagina, cervix, mammary, thyroid, adrenal and pituitary glands of PND 139 animals.
2-Generation Reproduction - Rat	00150557	In the F0 generation, no apparent adverse effect was observed on fertility. Pre-coital intervals were comparable among the groups. The duration of gestation was increased (0.6 days) at the high-dose in F0 rats producing the F1b pups. The gestation survival index was comparable among the groups for the F1a pups but was significantly decreased for the F1b litters. There was a significant decrease in the number of F1a female fetuses at the high-dose. The number of F1b pups born dead/dying by PND 1 was significantly increased at the high-dose. Fl a litter size was slightly lower at the high-

Table 11. Evaluation of Data Submitted in Relation to the Uterotrophic Assay		
Chemical: 2,4-D		**PC Code: 031001**
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		dose, but F1b litter size was significantly lower than the control. Fla pup viability was comparable throughout weaning, but the Flb pup viability was significantly lower throughout the weaning period. There was a significant decrease in Flb pup survival to lactation day 4 at the high-dose level as well as survival to lactation day 28. In the F1 generation, no apparent adverse effect was observed on fertility at either dose level. Pre-coital intervals and gestation lengths were comparable among the groups. The gestation survival index and the viability index were comparable among the groups for both the F2a and F2b litters. Litter size, body weights, and the sex ratio were comparable among treatment groups in both the F2a and F2b litters. No treatment-related histopathological lesions were seen in the ovaries of offspring of any generation.
Developmental Toxicity- 2,4-D Acid -Rat	00130407	No treatment-related changes were seen in pregnancy rate, number of corpora lutea, mean number of implantations/litter, post-implantation loss, early or late resorptions, number of live fetuses/litter, fetal sex ratio, or soft tissue abnormalities at any dose.
Developmental Toxicity- 2,4-D Salt & Esters – Rat [Charles *et al.*, (2001)]	45761204	No treatment-related changes were seen in pregnancy rate, number of corpora lutea, mean number of implantations/litter, post-implantation loss, early or late resorptions, number of live fetuses/litter, fetal sex ratio, or soft tissue abnormalities for 2,4-D acid or any of its amine salts (2,4-D DMA; 2.4-D DEA; 2.4-D IPA; 2.4-D TIPA) or esters (2.4-D BEE, 2.4-D EHE and 2,4-D IPE).
Developmental Toxicity- 2,4-D Acid -Rabbit	41747601	No treatment-related changes were seen in pregnancy rate, number of corpora lutea, mean number of implantations/litter, post-implantation loss, early or late resorptions, number of live fetuses/litter, fetal sex ratio, or soft tissue abnormalities at any dose.
Developmental Toxicity – 2,4-D Salt & Esters - Rabbit [Charles *et al.*, (2001)]	45761204	No treatment-related changes were seen in pregnancy rate, number of corpora lutea, mean number of implantations/litter, post-implantation loss, early or late resorptions, number of live fetuses/litter, fetal sex ratio, or soft

Table 11. Evaluation of Data Submitted in Relation to the Uterotrophic Assay		
Chemical: 2,4-D		**PC Code: 031001**
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		tissue abnormalities for 2,4-D acid or any of its amine salts (2,4-D DMA; 2.4-D DEA; 2.4-D IPA; 2.4-D TIPA) or esters (2.4-D BEE and 2.4-D EHE).
Chronic Toxicity /Carcinogenicity – Rat	43612001	No treatment-related changes were seen in T3 concentrations at any dose. Thyroxin (T4) levels were significantly ($p < 0.05$) decreased in females at all intervals measured (6, 12, 18 and 24 months). Ovarian weight was decreased at the high dose at 12 (9%) and 24 (42%) months and at 75 mg/kg/day (35%) at study termination. Absolute and or thyroid weights were significantly ($p < 0.05$) increased at the mid (75 mg/kg/day) and high (150 mg/kg/day) dose groups at 12 and 24 months. No treatment-related histopathological lesions were seen in the ovaries, uterus, vagina, cervix, mammary, adrenal and pituitary glands. Decreased secretory material in the thyroid follicular epithelial cells was seen in 8 females at 150 mg/kg/day compared to none in the controls.
Carcinogenicity – Female Mouse	43879801	Female reproductive organ weights were not evaluated. No treatment-related histopathological lesions were seen in the uterus, ovaries, oviduct, vagina, cervix, mammary, thyroid, adrenal or pituitary glands.
1981: Subchronic Oral Toxicity - Rat	00102451	Total T4 levels were significantly ($p<0.05$) decreased 50% and 72% at the 100 and 150 mg/kg/day dose groups, respectively. Organ weights were not evaluated. No treatment-related histopathological lesions were seen in the ovaries, uterus, mammary thyroid, adrenal or pituitary glands.
1991: Subchronic Oral Toxicity- 2,4-D Acid - Rat	41991501	Thyroid hormone levels (T3 and T4) were decreased at the mid (100 mg/kg/day) and high (300 mg/kg/day) dose groups at 6 and 13 weeks. At 6 weeks, T3 levels were decreased (87% not significant) and T4 levels were significantly ($p<0.05$) decreased (30%). At 13 weeks, T3 levels were significantly ($p<0.05$) decreased (66%) and T4 levels were significantly ($p<0.05$) decreased (42%). Absolute and relative thyroid weights were significantly ($p<0.05$) increased (168% and 126%) at the high dose. Absolute and relative ovarian weights were significantly ($p<0.05$) increased (58% absolute and 82% relative) at the high dose. Treatment-related

Table 11. Evaluation of Data Submitted in Relation to the Uterotrophic Assay		
Chemical: 2,4-D		**PC Code: 031001**
890.1600 - Uterotrophic Assay (Rat)		
		histopathological changes were observed primarily in the high-dose group and included: hypertrophy of the zona glomerulosa of the adrenal cortex in 10 of 10 females at the mid and high dose groups and hypertrophy of the thyroid glands in 8 of 10 females at the high dose compared to 3 of 10 control females.
Subchronic Oral Toxicity – 2,4-D Salts & Esters – Rat [Charles *et al.*, (1996)]	45761213	No treatment-related changes in absolute or relative weights of the ovaries, adrenals or pituitary glands. Histopathology revealed hypertrophy of the adrenal cortex at the high dose with the acid (10/10); 2,4-D DMA salt (9/10); and 2,4-D 2-EHE (10/10). No treatment-related histopathological lesions were seen in the ovaries, mammary glands, thyroid and pituitary glands.
Subchronic Oral Toxicity -Mouse	41991502	No treatment-related changes in absolute or relative weights of the ovaries, thyroid, adrenals or pituitary glands. No treatment-related histopathological lesions were seen in the uterus, ovaries, thyroid, adrenal or pituitary glands.
Subchronic Inhalation Toxicity- Rat	47398701	No treatment-related changes in absolute or relative weights of the ovaries, uterus and adrenals glands. No treatment-related histopathological lesions were seen in the ovaries, uterus, thyroid, adrenal and pituitary glands.
Avian Reproduction Study with Bobwhite Quail (*Colinus virginianus*)	45336401	Bobwhite quail were exposed to 2,4-D at 0 (control), 147, 382, and 962 mg a.i./kg diet (mean-measured). No treatment-related adverse effects were determined from 2,4-D exposure on reproduction, growth, or survival endpoints up to 962 mg a.i./kg diet.
Epidemiology Studies (PETA OSRI)	N/A	See discussion below in Section 3.

3. Agency's Evaluation of the OSRI:

The submission provided by the Test Order Recipient stated that "The primary purpose of the Uterotrophic Assay is to identify estrogenic compounds. It looks for evidence of uterine stimulation (uterine engorgement) in either immature or ovariectomized females. As noted above 2,4-D showed no consistent evidence of binding to or interacting with the ER (including ERα or ERβ) in multiple *in vitro* studies, including data developed for ToxCast® under the auspices of the US EPA. The vast majority of the multiple in vitro studies were negative. There were no findings in the regulatory toxicology data base for 2,4-D suggesting an estrogenic effect in females, even at doses exceeding the KMD. As shown

Table 11. Evaluation of Data Submitted in Relation to the Uterotrophic Assay	
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890.1600 - Uterotrophic Assay (Rat)

in the Uterotrophic Assay matrix in Appendix II and discussed in the text, 2,4-D was evaluated in two reproductive toxicity studies including an F1-extended one-generation reproductive toxicity study (Marty *et al.,* 2010) incorporating multiple endocrine-related endpoints sensitive to estrogenicity. These evaluations included estrous cyclicity assessment (in both parental females and F1 offspring), time to vaginal opening (including notation of retained vaginal threads), anogenital distance, quantitative ovarian follicle count, reproductive organ weight evaluations and detailed histopathology of female reproductive organs across multiple life stages. Further, the uterus was evaluated both grossly and histopathologically in PND 28 female weanlings (Rodwell and Brown, 1985), providing an evaluation of potential estrogenic uterine growth and/or stimulation in immature non-cycling females, corresponding to the alternate (pre-pubertal non-ovariectomized) version of the uterotrophic assay. No effects suggesting estrogenicity of 2,4-D were evident in these studies. There was also no exposure related increase in mammary tumors in either the rat or female mouse oncogenicity studies. These multiple endpoints are sensitive to estrogenic and/or anti-estrogenic modes of action (MOA), and showed no evidence of exposure related effects. High dose effects of 2,4-D was seen on pup growth and survival; these findings were restricted to maternally systemically toxic doses that exceeded the renal clearance saturation threshold, and there was no indication they were estrogen-mediated. One high dose report in the published literature (Evangelista deDuffard *et al.,* 1990) claims 2,4-D disrupted the estrous cycle in rats; this report is an abstract only and too few data are available to evaluate the basis for this observation. The single dose level administered in that study was, in so far as it was accurately characterized, well above the KMD."

"An avian reproductive toxicity study in bobwhite quail failed to show any evidence of estrogenic or anti-estrogenic effects (Mitchell et al., 1999). One study in the published literature reported vitellogenin induction in immature trout exposed to 2,4-D (Xie *et al.,* 2005); this study suffered from significant endpoint response variability and the findings were inconsistent with in vitro studies suggesting a lack of ER binding or activation in trout (Petit *et al.,* 1997). The Fish Short-Term Reproduction assay that the 2,4-D Task Force plans to conduct will further characterize whether 2,4-D has any estrogenic effects in fish. In *toto*, the potential estrogenicity of 2,4-D has been thoroughly characterized, and there is no need for a Uterotrophic Assay. Based on the extensive data available and submitted as OSRI, the 2,4-D Task Force requests a waiver from a Uterotrophic Assay."

The PETA comments submitted to the Agency in response to the EDSP screening order issued for 2,4-D stated that "In summary, 2,4-D is an extremely well-studied chemical. Its endocrine disrupting potential has already been addressed in mammalian subchronic, reproductive and developmental studies as well as in numerous in vitro assays, in chronic studies in birds, fish and reptiles and in epidemiologic studies. Further, a study designed in consultation with the EPA to definitively address remaining endocrine-sensitive endpoints is nearing completion. There is no need for further testing under the EDSP."

Table 11. Evaluation of Data Submitted in Relation to the Uterotrophic Assay	
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890.1600 - Uterotrophic Assay (Rat)

On review of the OSRI submitted, the Agency made the following observations with regard to the estrogen pathway:

In the Extended One-Generation Reproduction Study:

- Reproductive indices were comparable among the groups [mating, fertility, time to mating, gestation length, pre-implantation loss, number of corpora lutea (GD17 dams), and ovarian follicle counts].
- There were no signs of dystocia in P1 dams and no adverse effects on post-implantation loss, litter size, and pup survival.
- There were no adverse effects on estrous cycle length or estrous cycle pattern, including a lack of persistent estrus [assessed in all P1 main study and GD 17 females and all F1 Set3 females].
- There was no treatment-related effect on developmental landmarks. AGD, which was measured in all pups, nipple retention (all non-culled pups), and age at vaginal opening (all Set 1-3 offspring) were comparable among the groups.
- In all three age groups in which uterine weights were measured [P1 (LD 22), F1 Set 1a (PND 70), and F1 Set 3 (PND 139)], increased absolute and relative uterine weights (10%-32%) were observed at the high dose.

The PETA submission provided a brief review of a few human studies that addressed potential reproductive and developmental toxicity of 2,4-D based on the exposure of Vietnam veterans to Agent Orange. The conclusion was that the veterans who had greater potential exposure to Agent Orange did not have an increased risk of fathering babies with major structural birth defects. The submission also stated that, even for pesticide applicators and the general population in an agricultural region of Minnesota, a more detailed cross-sectional analysis of this area showed no statistically significant correlation between 2,4-D use and excess adverse birth effects. The Agency believes that overall, prospective cohort studies still need to be conducted to confirm or test the hypothesis generated by these studies. In addition, additional detail is needed on exposures of the subjects to drugs and chemicals which may have an adverse effect on the male reproductive system that can contribute confounding effects in the outcome and interpretation of these data. While these data provide additional information that upon further investigation may contribute to hazard characterization, they do not provide confirmed or confident linkages between human exposure to 2,4-D and interaction with the estrogen pathway.

4. Conclusion: The requirement for the **Uterotrophic Assay (890.1600) is satisfied** based on the Extended One-Generation Rat Reproduction Study which provided numerous detailed measures of the endocrine system in developing offspring as a consequence of pre-natal and post-natal exposures to 2,4-D.

[1] -- = not measured; X = indicates the endpoint was measured in the assay but does not indicate whether or not it satisfies the data requirement of the test order. See section 3 below for a detailed explanation; N/A = not applicable

III. Summary of studies cited in the OSRI submission that were considered in the EDRT's evaluation

Chemical: 2,4-D		PC Code: 031001
MRID No.	**Citation in OSRI**	**Selected Endocrine Related Findings**
48161802	Study Type: ER Binding Assay Author: Blair *et al.* Year: 2000	Blair *et al.* (2000) tested 188 natural and xenochemicals (including 2,4-D) in an estrogen receptor (ER) assay using rat uterine cytosol as the receptor source. Test chemicals were initially tested at two high concentrations. Positive chemicals were retested to obtain a concentration-response curve. 2.4-D was negative, but no data presented.
N/A	Study Type: Aromatase Study Author: Crain *et al* Year: 1997	The authors studied aromatase activity in the gonadal-adrenal-mesenephros complex taken from female alligators which had been exposed *in ovo* to various chemicals (including 2,4-D) to investigate the mechanism by which a pesticide contaminated lake affected sex-determination. Exposure to 2,4-D did not affect aromatase. This assay measures the effect of a chemical on the induction of aromatase in hatchlings due to *in ovo* exposure.
N/A	Study Type: Aromatase Study Author: Crain *et al* Year: 1999	Crain *et al.*, (1999) studied hepatic aromatase activity using liver slices from hatchling alligators with androstenedione as the substrate and tritiated water as the measured reaction product. This experiment determined the effect of pesticides on aromatase levels in tissue when hatchlings were exposed *in ovo*. It was reported that 2,4-D had no affect on aromatase levels in this study. Similar to the Crain *et al.*, 1997 paper, this study appears to focus on the effect of a chemical on the induction of aromatase in hatchlings due to *in ovo* exposure.
48074110	Study Type: AR Binding Assay Author: Fang *et al.* Year: 2003	In this study, the authors tested 202 natural, synthetic and environmental chemicals (including 2,4-D) for the potential to bind to the androgen receptor (AR) using a recombinant AR binding assay. Data for each competitor and R1881 (standard AR ligand) standard curve were plotted as [^{3}H]R1881 bound (relative to the standard) vs. molar concentrations. 2,4-D was adequately tested at concentrations from 4.28×10^{-9} to 4.28×10^{-4} M. All assays were run in duplicate with at least two replications. Appropriate reference chemicals were used in the study. The IC_{50} (50% inhibition of R1881 standard) values were reported and the relative binding affinities (RBA) were calculated for each chemical. Based on the RBA values, the chemicals were

Chemical: 2,4-D		PC Code: 031001
MRID No.	**Citation in OSRI**	**Selected Endocrine Related Findings**
		classified as strong binders, moderate binders, weak binders or inactive (non-binders). 2,4-D was classified in this study as a non-binder. The authors compared the recombinant AR binding data generated in this study to a published data set that was generated using the rat ventral prostate cytosol AR method similar to the one used in the EDSP Tier 1 assay. There were 20 chemicals in common between the two data sets. The results from the two methods were generally comparable with the recombinant AR method demonstrating adequate sensitivity to identify strong, moderate and weak androgen receptor binders. Radiolabeled [^{3}H]-R1881 was used to measure competitive AR binding (similar to the Tier 1 assay). No concentration response information were reported for 2, 4-D.
48279302	Study Type: Thyroid function Author: Florsheim and Velcoff (1962) Species: Rat Strain: Sprague Dawley Sex: Male Age at Initiation: Young adult	Study design: Male Sprague-Dawley rats on a restricted iodine diet regimen for 26 days with a daily subcutaneous injection of 1 cc iodine solution (0.8 µc I131 and 5 or 10 µg stable iodide). Experimental rats were subcutaneously injected with 80 mg/kg/day 2,4-D for the last 7 days of the regimen. After autopsy, a 3 cc serum sample from each animal was evaluated. Results: A significant increase in 24-hour iodine-131 (I131) uptake and significant decreases in serum protein-bound-iodine (PBI) and thyroid:serum radioiodide ratio were observed. However, 2,4-D exposure had no effect on serum or pituitary TSH concentrations, thyroidal cell height, or thyroid histopathology. Therefore, these findings provide a possible mechanistic explanation for decreases in circulating thyroid concentrations in rats at high dosages of 2,4-D, but do not provide evidence of a biologically significant adverse effect.
48279303	Study Type: Thyroid function Author: Florsheim *et al.*(1963) Species: Rat Strain: Sprague Dawley	Study design: This study evaluated the distribution of thyroxine after 2, 4-D exposure. Male Sprague-Dawley rats were maintained on a restricted iodine diet regimen with fixed dose iodine replenishment and dosed subcutaneously with 80 mg/kg/day 2,4-D for the last 7 days of the regimen Results: A significant increase in 24-hour I131 uptake and significant decreases in serum protein bound iodine (PBI) and thyroid: serum radioiodide ratios were

Chemical: 2,4-D		PC Code: 031001
MRID No.	**Citation in OSRI**	**Selected Endocrine Related Findings**
	Sex: Male Age at Initiation: Young adult	observed. A 50% decrease in protein-bound iodine was reported with subcutaneous administration of 80 mg/kg/day 2,4-D to male rats for 7 days. However, 2,4-D exposure had no effect on serum or pituitary TSH concentrations, thyroid follicular cell height, or thyroid histopathology. The authors concluded that "2,4-D reduces the serum binding capacity for thyroxine" and "2,4-D lowers serum thyroxine binding by competing with thyroxine for its binding sites, although it appears to be a weak competitor."
48242003	Study Type: Estrogen Receptor Activation Assay Author: Hurst and Sheahan Year: 2003	Hurst and Sheahan tested 26 pesticidal chemicals (including 2,4-D) for estrogenic activity using a Yeast Estrogen Screen (YES). The yeast cells used in this assay contain an integerated human estrogen receptor (hER) gene and carry a plasmid containing estrogen responsive elements (EREs) controlling the expression of a LacZ reporter. The results were reported to be negative for 2,4-D.
N/A	Study Type: ER Antiestrogenicity Study *In Vitro.* Author: Jung *et al.* Year: 2004	The anti-estrogenic activity of 52 chemicals (including 2,4-D) were evaluated using the following approach. Chemicals were first tested for antiestrogenicicty in the yeast two hybrid system transfected with a rat ER receptor. Chemicals that were identified as antiestrogens in this assay were then tested in a transcriptional activation assay using MCF-7 cells, and an ER binding assay using fluorescence polarization. This study has several disadvantages. First the gatekeeper was the yeast assay for antiestrogenic activity, so no additional testing would be conducted if a chemical were not identified as an antiestrogen by the yeast assay. Because 2,4-D was not positive in the yeast assay it appears that it was never tested in the binding assay, but the article is not absolutely clear on this point. No ER binding data were reported for 2,4-D.

Chemical: 2,4-D		PC Code: 031001
MRID No.	**Citation in OSRI**	**Selected Endocrine Related Findings**
N/A	Study Type: ER Transcriptional Activation Author: Jungbauer and Beck Year: 2002	The authors (2002) tested 2,4-D in yeast cells expressing human estrogen receptor α and a reporter plasmid containing the LacZ gene under the control of the vitellogenin hormone response element. 2,4-D was reported to be negative; however the concentrations were not identified nor were data provided.
N/A	Study Type: AR Binding Assay Author: Kim *et al.* Year: 2005	The authors conducted a competitive AR receptor binding assay using a whole cell assay in monkey kidney COS-1 cells transiently transfected with human AR. The results indicated that 2,4-D had some affinity for androgen receptor, although it was lower than dihydroxytestosterone. The authors state that the possibility of the androgenic effect of 2,4-D may be independent of AR binding cannot be exclude.
48033008	Study Type: ER and AR Transcriptional Activation Assays Author: Kojima *et al.* Year: 2004	In this study, Kojima *et al.*, tested 200 chemicals (including 2,4-D) for agonism and antagonism to human estrogen receptor (ER α and ERβ) and human androgen receptor (AR) using Chinese Hamster Ovary-K1 cells in luciferase reporter assay. The relative activity for each pesticide tested was expressed as REC_{20} (20% relative effective concentration), which is the concentration of the test compound showing 20% of the activity of 10^{-10} M E2, 10^{-9} M E2 or 10^{-9} M DHT for ERα, ERβ or AR, respectively. Similarly, RIC_{20} (20% relative inhibitory concentration) was reported as the test material concentration showing 20% inhibition of the activity induced by 10^{-11} M E2, 10^{-10} M E2, or 10^{-10} M DHT for ERα, ERβ or AR, respectively. The authors concluded that 2,4-D tested at concentration range up to 10^{-5} M, had neither agonist nor antagonist activity for ER or AR. No concentration data were prsented. The authors stated that the chemicals were tested at non-cytotoxic concentrations (data not shown).

Chemical: 2,4-D		PC Code: 031001
MRID No.	**Citation in OSRI**	**Selected Endocrine Related Findings**
N/A	Study Type: Yeast-Two Hybrid Assay Author: Lee *et al.* Year: 2006	Lee *et al.* compared several combinations of "bait and fish" components of yeast-two hybrid systems to detect estrogenic activity. A selection of compounds (including 2,4-D) were analyzed. 2,4-D was reported to be positive in this system with binding over the range 2.09×10^{-4} to 5.42×10^{-6} M.
48041723	Study Type: ER Transcriptional Activation Assay Author: Lemaire *et al.* Year: 2006	This study tested 49 pesticides (including 2,4-D) for ERα and ERβ activity in HeLa cells stably transfected with a luciferase reporter gene. Substances were tested first at 10 μM. Positive substances were re-tested to determine a dose response curve and calculate an EC50. 2,4-D was reported negative with a percent activity in ERα and ERβ of 8.4 ± 1.4 and 10.2 ± 2.1 which was not statistically different from the solvent DMSO (9.3 ± 1.3 and 10.8 ± 1.6).
48033008	Study Type: Cell Proliferation Assay Author: Lin and Garry Year: 2000	In this study, 16 agricultural chemicals (including 2,4-D) were evaluated for estrogen responsive proliferation using MCF-7 breast cancer cell line. The commercial grade 2,4-D induced cell proliferation at 1 and 10 μg/ml, whereas the technical grade 2,4-D failed induce a proliferative response over the same concentration range.
48041727	Study Type: Yeast-Two Hybrid Assay Author: Nishihara *et al.* Year: 2000	The authors tested 2,4-D for estrogenic activity in a yeast two hybrid assay. It was tested up to 10^{-3} M and found to be negative. The limitations of yeast –based assays were discussed in the paper. Nishihara notes that his test system may give false negative results for various reasons including the following: 1) some act *via* receptors other than ERα, 2) some involve a pathway other than *via* receptor-mediated gene expression, 3) some act as antagonists, 4) some act after being metabolized by animal cells, 5) some have inhibitory activity against the galactosidase assay and biocidal activity against the yeast cell, and 6) some cannot be transported into the cell, resulting in cellular concentrations below the sensitivity level.

Chemical: 2,4-D		PC Code: 031001
MRID No.	**Citation in OSRI**	**Selected Endocrine Related Findings**
48041727	Study Type: AR and ER Transcriptional Activation Assays Author: Orton *et al.* Year: 2009	In this study, 12 environmental chemicals (including 2,4-D) were screened for AR and ER activation using recombinant yeast assays (YAS and YES assays, respectively). 2,4-D was negative in these assays for (anti-) estrogenic and (anti-) androgenic activity over the concentration range tested (0.01- 1000 μM).
48074102	Study Type: ER Binding, ER Transcriptional Activation and Vitellogenin mRNA Expression Assays. Author: Petit *et al.* Year: 1997	In this study 2,4-D was tested in three different *in vitro* assays. It was tested in yeast a transactivation assay over a range of 10^{-8} to 10^{-4} M in which the yeast was transfected with rainbow trout ERα using a β-galactosidase reporter. Similarly a competitive binding assay was run using yeast cells and rainbow trout ERα. It was also tested for its ability to induce vitellogenin mRNA in a primary rainbow trout hepatocyte culture. The assay results were reported as follows: β-Gal expression in the TA assay = 17.95%, 8.0% Vg induction, and fold induction needed to displace 50% of bound E2 > 10,000. All results were described as negative
N/A	Study Type: Aromatase Study Author: Spiteri *et al.* Year: 1999	This study investigated the role of *in ovo* exposure to herbicides (including 2,4-D) on hepatic aromatase activity in alligators. Liver aromatase activity was measured using a tritium release method. This assay measures the effect of a chemical on the induction of aromatase in hatchlings due to *in ovo* exposure. Exposure to 2,4-D did not affect aromatase.
N/A	Study Type: Cell Proliferation Assay Author: Soto *et al.* Year: 1995	The authors state the purpose of this study was to validate the E-Screen (MCF-7 cell proliferation assay) by testing the estrogenicity of a number of environmental chemicals (including on 2.4-D). In this assay, 2,4-D failed on induce a proliferative response in MCF-7 cells. No data were provided to substantiate this conclusion.

Chemical: 2,4-D		PC Code: 031001
MRID No.	**Citation in OSRI**	**Selected Endocrine Related Findings**
48161802	Study Type: ER Binding Assay Author: Vonier *et al.* Year: 1996	2,4-D was tested in a competitive binding assay using ER derived from alligator oviducts. 2,4-D was reported to be a non-binder, but no data were shown to substantiate this conclusion.
N/A	Study Type: Vitellogenin Assay Author: Xie *et al.* Year: 2005	In this study, the estrogenic activities of four herbicides (including 2,4-D) were evaluated using a rainbow trout vitellogenin assay. Juvenile trout were exposed to 2,4-D for 7 days. Although positive findings were reported, this study suffered from significant endpoint response variability.
47972101	Study Type: Extended One-Generation Reproduction Classification: Acceptable Year: 2010 Species: Rat Strain: Crl:CD (SD) BR Sex: Male and Female Age at Initiation: P=10 weeks	Dose levels tested: 0, 5, 15 and 30 (females) and 40 (males) mg/kg/day in the diet Dosing regimen: Rats received the test diet for approximately four weeks prior to mating and continuing through mating (up to 2 weeks), gestation (3 weeks), and lactation (3 weeks). Exposure of P1 males continued for 7 weeks from the initiation of the mating phase. P1 females were exposed until lactation day (LD) 22 (end of lactation). A satellite group of P1 females (12/dose) were subject to the same exposures as the P1 females on the main study (exposure for 4 weeks during the pre-mating, up to 2 weeks during the mating period and during gestation until termination on gestation day 17 (GD 17). Satellite males were not exposed to dietary 2,4-D except during co-housing with satellite females during the mating period. Study Design: P1 Generation: A comprehensive evaluation of P1 male and P1 female reproductive system were conducted, including an evaluation of gonadal function, the estrous cycle, sperm parameters, mating performance, conception, gestation, parturition and lactation, as well as survival, growth and development of the offspring. Selected systemic toxicity parameters were also evaluated in the P1 males and P1 females.

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		Satellite GD 17 Females: A satellite group of P1 females (12/dose) was included for assessments of selected systemic toxicity parameters, clinical chemistry/hematology, thyroid hormone levels, thyroid weights, plasma 2,4-D levels, histopathology, and selected reproductive parameters during gestation (corpora lutea and implantation numbers). F1 Generation: F1 offspring were evaluated for potential effects on the reproductive and endocrine systems, thyroid function, and other systemic toxicity parameters. In-life parameters in all F1 offspring included anogenital distance, nipple retention and puberty onset. Selected F1 offspring were divided into three different groups (Sets 1, 2, and 3) at weaning (PND 22). Each set of F1 offspring was maintained on the test diet until PND 60 (Set 1b F1 offspring), ≈PND 70 (Sets 1a and 2a F1 offspring), or ≈PND 90 (Sets 2b and 3 F1 offspring). • Set 1a (10/sex/dose): assessment of general systemic and thyroid toxicity, which included clinical chemistry/hematology parameters, thyroid hormone (T3, T4 and TSH) assessment, and urinalysis (males only). Post-mortem evaluations in Set 1a (PND70) included gross pathology, organ weights and histopathology on a wide range of tissues, including thyroids. • Set 3 (23-27/sex/dose): assessment of reproductive/endocrine toxicity, which included estrous cycle evaluation and post-mortem evaluations that focused on reproductive organs, sperm assessment, and ovarian follicle counts on PND 139. TK analyses were conducted on Set 3 males and females on PND 63 and 84 to determine plasma 2,4-D levels. In addition, selected pups culled on PND 4 were used to assess thyroid hormone levels. Additional data were gathered from F1 offspring not assigned to Sets 1-3. On PND 22, unselected weanlings were either perfused for examination of neuropathology (12/sex/dose) or euthanized for assessment of systemic toxicity, which included thyroid hormone assessment, organ weights, and post-mortem examinations (gross pathology and histopathology) in 10/sex/dose.

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		Results: P1 Adult Rats: • Male and female mating, conception, fertility, and gestation indices were comparable among the groups, and post-implantation loss was comparable among the groups. Both the time to mating and gestation length were comparable among the groups. • No alterations were observed in the estrous cycle pattern of F1 females. • No differences were seen in mean estrous cycle length in P1 females. • No treatment-related effects were observed on sperm motility or progressive motility. • No treatment-related effects were seen in testicular spermatid and epididymal sperm counts or in morphology (i.e., the proportion of abnormal sperm). The decreased reproductive and accessory sex gland weights described below were determined to be not treatment related since the values in the concurrent control were outside of the range of the values seen in the testing laboratories historical control data. • Testes: Although statistical significance was not attained, there was a dose-dependent decrease in absolute and relative testicular weights with the decreases reaching ↓5% for absolute and ↓8% for relative at the high dose. • Epididymides: Absolute (↓5%) and relative (↓8%) weights were decreased at the high dose; not statistically significant. • Seminal vesicles: Although statistical significance was not attained, there were dose-dependent decreases in absolute and relative weights at the mid and high dose groups: ↓12% for absolute and ↓12% for relative at the mid-dose and ↓12% for absolute and ↓14% for relative at the high dose.

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		• Prostate: Although statistical significance was not attained, there were dose-dependent decreases in absolute and relative weights at the mid and high dose groups: ↓9% for absolute and ↓8% for relative at the mid-dose and ↓10% for absolute and ↓11% for relative at the high dose. • Uterus: Absolute (↑17%) and relative (↑17%) weights were decreased at the high dose; not statistically significant. • No treatment-related changes were seen in the absolute or relative weights of the ovaries, thyroid, adrenal or pituitary glands. • No treatment-related histopathological lesions were seen in the testes, epididymides, seminal vesicles, prostate, ovaries, oviducts, uterus, vagina, cervix, mammary, thyroid, adrenal and pituitary glands. GD 17 Satellite Females: • Reproductive indices and the numbers of corpora lutea and implantations were comparable among the groups. • There was a slight increase in resorptions at the high dose (0.9±1.1) compared to controls (1.5±1.7); although there was wide variability (standard deviations exceed the means). • There was a slight increase in post-implantation loss at the high dose (9.22±12.67) compared to controls (5.54±6.94); although there was wide variability (standard deviations exceed the means). • Absolute thyroid weight was increased (↑9%) at the low and high dose groups, but not at the mid dose.

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		• Although statistical significance was not attained, decreases in thyroid hormone levels were seen in females at the mid dose (T3,↓5% and T4,↓8%) and at the high dose (T3,↓7%, T4,↓9% and TSH,↑25%) dose groups; • Histopathology of the thyroid glands revealed smaller follicles (colloid resorption) in 3/12 females (graded as very slight) vs. 0/12 in the controls. <u>F1 Offspring Set 1 (PND 70)</u> • No treatment-related effects were seen on the numbers of live or dead F1 pups born/litter or on pup survival or sex ratio. • No treatment-related difference in absolute or relative anogenital distance in either sex. • No differences in nipple/areolae retention between control and high-dose groups in either sex. • There was a slight delay in preputial separation (1.6 days), which was accompanied by a very slight reduction in body weight compared to the control (↓2.1 grams; 99% of control) at the high dose. . This finding was not considered to be biologically relevant. • The age at vaginal opening was comparable among the groups of F1 females. • In the pups culled on PND 4, there were no statistically-significant differences in serum T3, T4, or TSH levels. T3 level was decreased in males only at the high dose (↓7%) and in females at the low (↓8%) and mid (↓13%) dose groups but not at the high dose. T4 was reduced to a similar extent in both sexes at the mid- (↓14%-15%) and high (↓12%-14%) dose levels, and TSH level was increased (↑19%) at the high dose.

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		• In the PND 22 pups, males displayed reductions in T3 concentrations at the mid (↓19%, $p < 0.05$) and high (↓13%) doses, but there was no dose response. T4 was decreased (↓28%) only at the high dose. Female pups displayed a non-statistically significant reduction (↓20%) in T4 at the high dose. • Among PND 62/64 day offspring, T3 levels were comparable between the treated and control groups. In males, T4 levels were increased at the mid dose (↑12%) and decreased (↓13%) at the high dose. In females, T4 levels were increased (↑19%) at the mid and high dose groups. TSH levels were increased in males at the mid (↑26%) and high (↑23%) dose groups and in females at the mid (↑1%) and high (↑24%) dose groups. • In the PND 70 offspring, although statistical significance was not attained, treatment-related changes observed in organ weights were: decreases in the absolute weights of the prostate (↓6%), epididymides (↓6%) and the thyroid (↓11%), adrenal (↓12%) and the pituitary (↓14%) glands in males at the high dose, and the increases in the absolute ovarian (↑9%) and uterine weights (↑31% absolute and ↑32% relative) in females at the high dose. • No treatment-related histopathological lesions were seen in the testes, epididymides, seminal vesicles, prostate, ovaries, oviducts, uterus, vagina, cervix, mammary, thyroid, adrenal and pituitary glands of PND 70 pups. <u>F1 Offspring Set 3(PND 139)</u> • No treatment-related differences were observed in mean estrous cycle length at any dose level compared to the control. There were no significant, treatment-related effects on the numbers of small follicles, growing follicles, or total follicles. • No treatment-related effects were seen on sperm motility or progressive motility, no differences in testicular spermatid and epididymal sperm counts, and no differences in the proportion of abnormal sperms.

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		• Absolute (↓9%) and relative (↓8%) pituitary gland weights were significantly lower in males at the high dose and the absolute (↓9%) and relative (↓10%) pituitary gland weights were non-significantly lower in females at the high dose. There was no associated histopathology in the pituitary glands. • Uterine weights were increased at the mid (↑10% absolute and ↑10% relative) and high (↑10% absolute and ↑11% relative) dose groups. • Ovarian follicle counts were comparable between the control and high dose females. • No treatment-related histopathological lesions were seen in the testes, epididymides, seminal vesicle, prostate, ovaries, oviducts, uterus, vagina, cervix, mammary, thyroid, adrenal and pituitary glands of PND 139 animals.
00150557 00163996	Study Type: Two-Generation Reproduction Classification: Acceptable Year: 1985 Species: Rat Strain: Fischer 344 Sex: Male and Female Age at Initiation: 5-6 weeks	Dose levels tested: 0, 5, 20 and 80 mg/kg/day in the diet for two consecutive generations. The F0 rats were fed the diet for 105 days; prior to mating and through gestation and lactation of two litters and for 30 days after weaning the last litter. The F1a litters were weaned at day 28 post partum. After a 2-week rest period, the F0 parental rats were re-bred using different male/female combinations to produce the Flb litters, from which 30 males/30 females/group were selected to become the F 1 parents. The Fl generation was administered the test material at target dose levels of 0, 5, and 20mg/kg/day [the high-dose level was dropped due to excess toxicity; there were an insufficient number of F lb pups] in utero and continuously via the milk or feed for 125 days postnatally and prior to mating and through gestation and lactation of two litters [F2a and F2b] and for 30 days after weaning the last litter.

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		Reproductive toxicity: • F0 Generation. No apparent adverse effect was observed on fertility. Pre-coital intervals were comparable among the groups. The duration of gestation was significantly increased in the high-dose [80 mg/kg/day] F0 females producing the F1b pups [22.5 days vs 21.9 days]. The gestation survival index was comparable among the groups for the F1a pups but significantly decreased for the F1b litters [31.7% vs 97.8%]. There was a significant decrease in the number of F1a female fetuses at the high-dose level [39% vs 54%]. The number of F1b pups born dead/dying by day 1 [110] was significantly increased at the high-dose level compared to the control [5]. Fla litter size was slightly lower at the high-dose level compared to the control [9.0 vs 10.1], but F1b litter size was significantly lower than the control [5.1 vs 9.5]. Fla pup viability was comparable throughout weaning, but the Flb pup viability was significantly lower throughout the weaning period. There was a significant decrease in Flb pup survival to lactation day 4 at the high-dose level [86.3%] compared to the control [100%] and other dose levels [98% and 99.6%], as well as survival to lactation day 28 [71.4% vs 100% (control) and other dose groups 99.4% and 100%]. • Fl Generation. No apparent adverse effect was observed on fertility at either dose level. Pre-coital intervals and gestation lengths were comparable among the groups. The gestation survival index and the viability index were comparable among the groups for both the F2a and F2b litters. Litter size, body weights, and the sex ratio were comparable among the groups in both the F2a and F2b litters. Organ weights: No treatment-related changes in absolute or relative weights of the testes or ovaries. Histopathology: No treatment-related histopathological lesions were seen in the testes or ovaries in the offspring of any generation.

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00130407	Study Type: Developmental Toxicity – 2,4-D Acid Classification: Acceptable Year: 1983 Species: Rat Strain: Fischer-344 Sex: Pregnant females Age at Initiation: 20 weeks	Dose levels tested: 0, 8, 25 and 75 mg/kg/day 2,4-D Acid in corn oil via gavage on days 6 through 15 of gestation; does were sacrificed on gestation day 20. Results: No treatment-related changes were seen in pregnancy rate, number of corpora lutea, mean number of implantations/litter, post-implantation loss, early or late resorptions, number of live fetuses/litter, fetal sex ratio, or soft tissue abnormalities at any dose.
45761204	Study Type: Developmental Toxicity – 2,4-D Salts and Esters Author: Charles *et al.* (2001) Year: 2001 Species: Rat Strain: Fischer 344 (acid) Sprague-Dawley (analogs) Sex: Pregnant females	This review article presents the data from the developmental toxicity studies conducted with the 2,4-D acid; 2,4-D dimethylamine salt (2,4-D DMA); 2,4-D diethylamine salt (2,4-D DEA); 2,4-D isopropylamine salt (2,4-D IPA); 2,4-D triisopropanolamine salt (2,4-D TIPA); 2.4-D butoxyethyl ester (2,4-D BEE); 2.4-D 2-ethylhextylester (2,4-D EHE); and 2,4-D IPE ester Dose levels tested: Test materials were administered via gavage daily on gestation days 6 through 15 at the following active ingredient (ai) and acid equivalent (ae) dose levels in mg/kg/day: 2,4-D acid (8, 25, and 75 ai); 2,4- DEA (15, 75, and 150 ai; 10.2, 50.8, 101.6 ae); 2,4-D DMA (15, 60.2 and 120.4 ai; 12.5, 50, and 100 ae); 2,4-D IPA (22, 65, and 190 ai; 17, 51 and 150 ae); 2,4-D TIPA (32.5, 100 and 325 ai; 17, 51 and 175 ae);

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	Age at Initiation: Time pregnant	2,4-D BEE (25, 75 and 185 ai; 17, 51 and 125 ae); 2,4-D EHE (15.1, 45.2 and 135.7 ai; 10, 30 and 90 ae); and 2,4-D IPE (12.3, 36.9 and 123 ai; 10, 30 and 100 ae). Separate control groups for each compound were administered the appropriate vehicle. Dams were sacrificed on gestation day 20. Results: No treatment-related changes were seen in pregnancy rate, number of corpora lutea, mean number of implantations/litter, post-implantation loss, early or late resorptions, number of live fetuses/litter, fetal sex ratio, or soft tissue abnormalities for any of the analogs.
41747601	Study Type: Developmental Toxicity Classification: Acceptable Year: 1990 Species: Rabbit Strain: New Zealand White Sex: Pregnant females Age at Initiation: 5 months	Dose levels tested: 0, 10, 30 and 90 mg/kg/day 2,4-D Acid in 0.5% aqueous methylcellulose by gavage on days 6 through 18 of gestation; does were sacrificed on gestation day 29. Results: No treatment-related changes were seen in pregnancy rate, number of corpora lutea, mean number of implantations/damn, post-implantation loss, early or late resorptions, number of live fetuses/dam, fetal sex ratio, or soft tissue abnormalities.

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MRID No.	**Citation in OSRI**	**Selected Endocrine Related Findings**
45761204	Study Type: Developmental Toxicity Author: Charles *et al* .,(2001) Species: Rabbit Strain: New Zealand White Sex: Pregnant females Age at Initiation: 5 - 7.5 months	This study evaluated the developmental toxicity of 2,4-D Amine Salts and Esters: 2,4-D DMA; 2.4-D DEA; 2.4-D IPA; 2.4-D TIPA ; 2.4-D BEE; & 2,4-D EHE. Dose levels tested: Test materials were administered via gavage daily either on GD 6 through 18 or GD 7 through 19.at the following ai and ae doses levels in mg/kg/day: 2,4-D acid (10, 30 and 90 ai); 2,4-D DEA (15, 30 and 60 ai;10.2, 20.3, 40.6 ae); 2,4-D DMA (12, 26.1 and 108.4 ai; 10, 30 and 90 ae); 2,4-D IPA (13, 38 an d95 ai; 10, 30 and 75 ae); 2,4-D TIPA (19, 56 and 140 ai; 10, 30 and 75 ae); 2,4-D BEE (15, 45 and 110 ai; 10, 30 and 75 ae); and 2,4-D EHE (15.1, 45.2 and 113.1 ai; 10, 30 and 75 ae). Dams were sacrificed on gestation day 28 or 29. Results: No treatment-related changes were seen in pregnancy rate, number of corpora lutea, mean number of implantations/litter, post-implantation loss, early or late resorptions, number of live fetuses/litter, fetal sex ratio, or soft tissue abnormalities for any of the analogs.

Chemical: 2,4-D		PC Code: 031001
MRID No.	**Citation in OSRI**	**Selected Endocrine Related Findings**
43612001	Study Type: Chronic Toxicity/ Carcinogenicity Classification: Acceptable Year: 1995 Species: Rat Strain: Fischer 344 Sex: Male and Female Age at Initiation: 7-8 weeks	Dose levels tested: 0, 5, 75 and 150 mg/kg/day in the diet for 104 weeks. Thyroid hormones: • No treatment-related changes were seen in T3 concentrations at any dose. • Thyroxin (T4) levels were decreased in both sexes at all intervals measured (6, 12, 18 and 24 months) with the decreases reaching statistical significance ($p < 0.05$) in males only at 12 and 24 months and in females at all intervals. • At 12 months, the decreases were -14% and -70% in males and -65% and -70% in females at the 75 and 150 mg/kg/day, respectively. • At 24 months, the decreases were -32% and -64% in males and -32% and -42% in females at 75 and 150 mg/kg/day, respectively. Organ weights: • No treatment-related changes were seen in absolute or relative adrenal weights. • Treatment-related changes were seen in the testes, ovaries and thyroid glands. Testes: • At 12 months, absolute testes weights were significantly ($p<0.05$) decreased (15%) at 150 mg/kg/day • At 24 months, absolute (52%) and relative (49%) testes weights were decreased at 150 mg/kg/day. Ovaries • Decreased at 150 mg/kg/day at 12 (9%) and 24 (42%) months and at 75 mg/kg/day (35%) at study termination. Thyroid • In males at 150 mg/kg/day, absolute thyroid weights were significantly ($p < 0.05$) increased at 12 (+19%) and 24 (+32%) months. • In males, at 150 mg/kg/day, relative thyroid weights were significantly ($p < 0.05$) increased 12 (+28%) and 24 (+40%) months.

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		• In females, at 75 mg/kg/day, absolute thyroid weights were significantly ($p < 0.05$) increased 12 (+22%) and 24 (+40%) months. • In females, at 75 mg/kg/day, relative thyroid weights were significantly ($p < 0.05$) increased 12 (+19%) and 24 (+66%) months. • In females, at 150 mg/kg/day, absolute thyroid weights were significantly ($p < 0.05$) increased 12 (+22%) and 24 (+32%) months. • In females, at 150 mg/kg/day, relative thyroid weights were significantly ($p < 0.05$) increased 12 (+35%) and 24 (+80%) months. Histopathology: No treatment-related histopathological lesions were seen in the epididymides, seminal vesicles, prostate, ovaries, uterus, vagina, cervix, mammary, adrenal and pituitary glands. • Atrophy of the testes was seen in 2/50 at 150 mg/kg/day compared to 0/50 in the controls. • Decreased secretory material, epithelial cells were seen in 8 females at 150 mg/kg/day compared to none in the controls.
43879801 43597201	Study Type: Carcinogenicity Classification: Acceptable Year: 1995 Species: Mouse Strain: B6C3F1 Sex: Male and Female Age at Initiation: 7-8 weeks	Dose levels tested: 0, 5.0, 62.5 and 125 mg/kg/day for males and 0, 5.0, 150.0 and 300.0 mg/kg/day for females in the diet for 104 weeks. Organ weights: Reproductive organ weights were not evaluated Histopathology: No treatment-related histopathological lesions were seen in the testes, epididymides, seminal vesicles, prostate, uterus, ovaries, oviduct, vagina, cervix, mammary, thyroid, adrenal or pituitary glands.

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00101588	Study Type: Subchronic Oral Toxicity Year: 1981 Species: Rat Strain: Fischer 344 Sex: Male and Female Age at Initiation: 6 weeks	Dose levels tested: 0, 15, 100 and 150 mg/kg/day 2,4-D Acid in the diet for 90-days. Additional groups of rats (3/sex/dose) were fed 0, 15, 60 and 100 mg/kg/day for 30 days for tissue analysis. Thyroid hormone: Total T4 levels were significantly ($p < 0.05$) decreased 50% and 72% at the 100 and 150 mg/kg/day dose groups, respectively. Organ weights: No treatment-related changes in absolute or relative testes weight were seen. Histopathology: No treatment-related histopathological lesions were seen in the testes, epididymides, accessory sex glands, prostate, ovaries, uterus, mammary thyroid, adrenal or pituitary glands.
41991501	Study Type: Subchronic Toxicity Classification: Acceptable Year: 1991 Species: Rat Strain: Fischer 344 Sex: Male and Female Age at Initiation: 6 weeks	Dose levels tested: 0, 1, 15, 100 and 300 mg/kg/day 2,4-D Acid in the diet for 90 days. Thyroid hormones: Decreased T3 and T4levels were observed at 100 and 300 mg/kg/day at 6 and 13 weeks in one or both sexes. • At 6 weeks, T3 levels were decreased in males (63% of control; $P<0.05$) and females (87% not significant) and T4 levels were significantly ($p < 0.05$) decreased in males (24%) and females (30%); • At 13 weeks, T3 levels were significantly ($p < 0.05$) decreased in males (73%) and females (66%) and T4 levels were significantly ($p < 0.05$) decreased in males (26%) and females (42%). Organ weights: Changes in absolute and/or relative organ weights (adrenals, testes with epididymides (males), ovaries (females), pituitary, thyroids/parathyroids (increased) were observed primarily at the high-dose level (both sexes). • Adrenals- the relative weight was significantly ($p < 0.05$) increased (130% of control) in males at the high dose while the absolute weight was decreased (64% of control) in females at this dose;

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		• Thyroids – the absolute and relative weights were significantly ($p < 0.05$) increased in males (140% absolute and 186% relative) and females (168% and 126%) at the high dose. • Testes – the absolute and relative weights were significantly ($p < 0.05$) increased (51% absolute and 67% relative) at the high dose. • Ovaries – the absolute and relative weights were significantly ($p < 0.05$) increased (58% absolute and 82% relative) at the high dose. Histopathology: Treatment-related histopathological changes were observed primarily in the high-dose group and included: • atrophy of the testes 8/10 vs. 0/10 in control males; • hypertrophy of the zona glomerulosa of the adrenal cortex in 8/10 males and 10/10 females at the mid and high dose groups vs. 0/10 in respective controls; and • hypertrophy of the thyroid gland in 8/10 females vs. 3/10 control females.
45761213	Study Type: Subchronic Toxicity Author: Charles *et al* Year: 1996 Species: Rat Strain: Fischer 344 Sex: Male and Female Age at Initiation: 5 weeks	This review article presents data from several rat subchronic toxicity studies conducted with 2,4-D acid; 2,4-D DMA, or 2,4-D 2-EHE. Dose levels tested: 0, 1, 15, 100, and 300 mg/kg/day (expressed as acid equivalent doses) in the diet for 90 days. Thyroid hormones: • T3 concentrations were significantly ($p < 0.05$) decreased in males at the high dose and in females at the mid and high dose groups with the acid and in males at the high dose and in females at the mid and high dose groups with the DMA salt; no data was provided for the EHE. • T4 concentrations were significantly ($p < 0.05$) decreased at the mid and high dose group in both sexes for the acid; in males at the high dose and in females at the mid and high dose groups with the DMA salt; and in males at the high dose and in females at the mid and high dose groups with the EHE.

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		Organ weights: • Thyroid - relative weights were significantly ($p < 0.05$) increased in males at the mid and high dose groups and in females at the high dose group with the acid; in both sexes only at the high dose with the DMA salt; and in both sexes at the high dose with EHE. • Testes – relative weights were significantly ($p < 0.05$) decreased in at the high dose for all three compounds. • No treatment-related changes in absolute or relative weights of the ovaries, adrenals or pituitary glands. Histopathology: • Hypertrophy of the adrenal cortex was seen at the high dose with the acid (8/10 males and 10/10 females); DMA (10/10 males and 9/10 females); and EHE (6/10 males and 10/10 females). • No treatment-related histopathological lesions were seen in the testes, epididymides, ovaries, mammary glands, thyroid and pituitary glands.
41991502	Study Type: Subchronic Oral Toxicity Classification: Acceptable Year: 1991 Species: Mouse Strain: B6C3F1 Sex: Male and Female Age at Initiation: 6-weeks	Dose levels tested: 0, 1, 15, 100, and 300 mg/kg/day in the diet for 90 days. Organ weights: No treatment-related changes in absolute or relative weights of the testes, ovaries, thyroid, adrenals or pituitary glands. Histopathology: No treatment-related histopathological lesions were seen in the testes, epididymides, uterus, ovaries, thyroid, adrenal or pituitary glands.

Chemical: 2,4-D		PC Code: 031001
MRID No.	**Citation in OSRI**	**Selected Endocrine Related Findings**
47398701	Study Type: Subchronic Inhalation Toxicity Classification: Acceptable Year: 2008 Species: Rat Strain: Sprague-Dawley Sex: Male and Female Age at Initiation: 8-9-weeks	Concentration tested: 0, 0.05, 0.10, 0.30 and 1.00 mg/L for 6 hours/day, 5 days/week for 28 days. Organ weights: No treatment-related changes in absolute or relative weights of the testes, ovaries, uterus and adrenals glands. Histopathology: No treatment-related histopathological lesions were seen in the testes, epididymides, seminal vesicles, prostate, ovaries, uterus, thyroid, adrenal and pituitary glands.
45336401	Study Type: Avian Reproduction Study Classification: Acceptable (formerly "Core") Year: 2000 Species: Northern Bobwhite Quail (*Colinus virginianus*) Sex: Males and females Age: Parental birds 21 weeks old at study initiation.	Exposure concentrations: 0 (control), 147, 382, and 962 mg a.i./kg diet (mean-measured). Reproductive parameters: • No statistically significant effects were determined for eggs laid, eggs set, viable embryos, live 3-wk embryos, normal hatchlings, 14-day old survivor, 14-day old survivor weight, eggshell thickness, and hatchling weight up to 962 mg a.i./kg diet • Statistically significant effects were determined for eggs cracked; however, the effects were not considered biologically significant Survival: • (P) No treatment-related mortalities were reported. Two incidental hen mortalities occurred at the 962 mg a.i./kg diet treatment level. One hen was found with a large head lesion during week 8 body weight procedure, and the other was inadvertently killed during the week 8 body weight procedure.

Chemical: 2,4-D		PC Code: 031001
MRID No.	**Citation in OSRI**	**Selected Endocrine Related Findings**
	Duration of exposure: Parents: 21 weeks Controls: No solvent or carrier	Necropsy: • (P) Gross pathological observations were made on head/foot/leg lesions, feather loss, spleen (small), liver (pale and mottled), small intestine (areas of hyperemia), gizzard (protruding growth), abdominal cavity (egg yolk peritonitis) Necropsies of dead or sacrificed birds showed no treatment-related mortalities, signs of toxicity, or treatment-related effects on adult body weight or feed consumption. In addition, no treatment-related gross abnormalities were observed at the 962 ppm a.i. level. Growth: • (P) No effects on male or female adult body weight were determined up to 962 mg a.i./kg diet treatment level Food Consumption • (P) No statistically significant effects on feed consumption was determined up to 962 mg a.i./kg diet treatment level birds. Overall: No treatment-related adverse effects were determined from 2,4-D exposure on reproduction, growth, or survival endpoints for bobwhite quail up to 962 mg a.i./kg diet.

IV. Studies cited in the OSRI but were not used in the EDRT's weight of evidence evaluations.

Study Type	MRID No.	Reason for not using the study
Early life stages of the fathead minnow	41737304	Study was classified as Invalid.
Erikson *et al,* (1984)	N/A	This case-study was designed to determine if Vietnam veterans exposed to Agent Orange were at an increased risk of fathering babies born with structural congenital malformations.
Evangelista de Duffard *et al.* (1990)	N/A	This study evaluated the behavioral changes in rats fed 2,4-D butyl ester. No endocrine-related endpoints were evaluated.
Evangelista de Duffard *et al.* (1995)	N/A	This study evaluated the altered behavioral responses in amphetamine challenged rats treated with 2,4-D. No endocrine-related endpoints were evaluated.
Hwang, U-G. (2002)	N/A	Only abstract is available in English.
Jungbauer and Beck (2002)	N/A	The concentrations were not identified nor were data provided.
Kim *et al,* 2002	N/A	This article is not in English.
Lamb *et a.,* (1981a)	N/A	This study in mice evaluated male mediated toxicity of mixtures containing 2,4-D, 2,4,5-T and TCDD.
Lamb *et a.,* (1981b)	N/A	This study in mice evaluated male mediated toxicity of mixtures containing 2,4-D, 2,4,5-T and TCDD.
Oakes *et al.* (2002)	N/A	This study evaluated a formulated product (Tordon®75; a 2,4-D and picloram combination) administered by gavage to rats.
Saghir *et al.* (2008a)	47417901	No endocrine-related endpoints were evaluated. This study identified the doses at which renal saturation resulted in non-linear pharmacokinetic performance as approximately 25 mg/kg/day for females and 65 mg/kg/day for males. These dose levels can be regarded as the Kinetically Derived Maximum Dose (KMD) for 2,4-D in the diet to rats.
Saghir *et al* (2008b)	47417902	No endocrine-related endpoints were evaluated. This study demonstrated the saturation of renal clearance and distinctly non-linear pharmacokinetics in male rats fed 100 mg/kg/day 2,4-D for 28 days.
Stoker *et al.* (2007)	N/A	Only an abstract is available.
Van den Berg *et al.*,1991	N/A	This study was conducted to determine the effect of selected chemicals (e.g., 2, 4-DB) on plasma thyroid hormone levels. 2, 4-D itself was not tested in the *in vivo* study.
USEPA (2005)	N/A	The studies cited by PETA in the Reregistration Eligibility Decision for 2,4-D have been rejected because it is unclear which studies and what forms of 2,4-D (*e.g.* acid, ester, salt) are being referred to in the OSRI.

V. Bibliography of Existing Data Cited in the OSRI

(i) Part 158 Studies

MRID	Citation
00101599	Gorzinski, S.J., Wade, C.E., Morden, D.C., Keyes, D.G., Dittenber, D.A., Kalnins, R.V., Schuetz, D.J. and Kociba, R.J. 1981b. Purified 2,4-Dichlorophenoxyacetic acid (2,4-D): results of a 13-week subchronic dietary toxicity study in the CDF Fischer 344 Rat. Unpublished study, Dow Chemical Company, Midland, MI.
00102451	Gorzinski, S.J., Wade, C.E., Morden, D.C., Keyes, D.G., Wolfe, E.L., Dittenber, D.A., Kalnins, R.V., Schuetz, D.J. and Kociba, R.J. 1981a. Technical grade 2,4-Dichlorophenoxyacetic acid (2,4-D): results of a 13-week subchronic dietary toxicity study in the CDF Fischer 344 Rat. Unpublished study, Dow Chemical Company, Midland, NI.
00130407	Rodwell, D.E. 1983. A teratology study in Fischer 344 rats with 2,4-dichlorophenoxyacetic acid – Final Report. Project No. WIL-81135. Unpublished study, WIL Research Laboratories, Inc., Ashland, OH.
00131303	Serota, D.; Colpean, B.; Burdock, G.; et al. (1983b) Subchronic Toxicity Study in Mice: 2,4-Dichlorophenoxy Acetic Acid (2,4-D): Project No. 2184-100. Unpublished study. Hazleton Laboratories America, Inc.
00131304	Serota, D.; Burns, C.; Burdock, G.; et al. (1983a) Subchronic Toxicity Study in Rats-2,4- Dichlorophenoxyacetic Acid (2,4-D): Project No. 2184-102. Unpublished study, Hazleton Laboratories America, Inc.
00150086	Serota, 1985 Addendum MRID.
00150557	Rodwell, D.E. and Brown, W.R. 1985. A Dietary Two-Generation Reproduction Study in Fischer 344 Rats with 2,4-Dichlorophenoxyacetic Acid: Final Report: Project No. WIL-81137. WIL Research Lab. Inc.
00163996	Rodwell, D.E. 1986. A dietary two-generation reproduction study in Fischer 344 rats with 2,4-Dichlorophenoxyacetic acid – addendum to the Final Report. Project No. WIL-81137. Unpublished study, WIL Laboratories, Ashland, OH.
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41586101 Culotta, J., Foster, J., Grimes, J., Hoxter, K.A., Smith, G.J. and Jaber, M. 1990a. 2,4-D (2,4-dichlorophenoxyacetic acid): a dietary LC50 study with the mallard. Unpublished study by Wildlife International Ltd. Project No. 103-307.

41735304 Schulze, G.E. 1990. 21-Day dermal irritation and dermal toxicity study in rabbits with 2,4-Dichlorophentoxyacetic acid. Project No. 2184-109. Unpublished study, Hazleton Laboratories America, Inc., Vienna, VA.

41737307 Vaishnav, D.D., Yurk, J.J. and Wade, B.A. 1990. 2,4-dichlorophenoxyacetic acid: acute toxicity to tidewater silverside (*menidia beryllina*) under flow-through conditions. ESE Project ID No. 3903008000-0210-3140, July 16, 1990. Unpublished study by ESE.

41737404 Mayes, M.A., Gorzinski, S.J., Potter, R.B. and Richardson, C.H. 1990. 2,4-Dichlorophenoxyacetic acid: evaluation of the toxicity to early life stages of the fathead minnow, Pimephales promelas rafinesque. Lab Report ES-2157. Unpublished study by The Dow Chemical Company. December 7, 1990.

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41991501 Schulze, G.E. 1991a. Subchronic toxicity study in rats with 2,4-Dichlorophenoxy-acetic acid. Project No. 2184-116. Unpublished study, Hazelton Laboratories America, Inc. Rockville, MD.

41991502 Schulze, G.E. 1991b. Subchronic toxicity study in mice with 2,4-Dichlorophenoxy-acetic acid. Project No. 2184-117. Unpublished study, Hazelton Laboratories America, Inc. Vienna, VA.

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43879801 Stott, W. (1995b) "2,4-Dichlorophenoxyacetic Acid: Dietary Oncogenicity Study in Male B6C3F1 Mice Two-Year Final Report". Lab Project Number K-002372-063MF. The Dow Chemical Company.

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